Inhibition of malignant glial cell growth by estramustine, an estrogen based antimicrotubule agent, and synthesized analogs of estrone, and the use of estramustine in combination chemotherapy

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Inhibition of malignant glial cell growth by estramustine, an estrogen based antimicrotubule agent, and synthesized analogs of estrone, and the basis for the use of estramustine in combination chemotherapy

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by

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For my grandparents,
Anne and Phil Weinstein

ABSTRACT

INHIBITION OF MALIGNANT GLIAL CELL GROWTH BY ESTRAMUSTINE, AN ESTROGEN BASED ANTIMICROTUBULE AGENT, AND SYNTHESIZED ANALOGS OF ESTRONE, AND THE BASIS FOR THE USE OF ESTRAMUSTINE IN COMBINATION CHEMOTHERAPY. Marc A. Weinstein, Joseph M. Piepmeier, David Keefe*, and Frederick Naftolin*. Section of Neurosurgery and *Department of OB/GYN, Yale University School of Medicine, New Haven, CT

The current treatment of malignant glioma has undergone no major changes over the past decade. The current regimen of surgery, radiation, and chemotherapy has not resulted in any significant alteration in the course of this disease. The development of new chemotherapeutic agents targeted against cellular structures specific in glioblastoma may offer some hope for patients stricken with this disease. We have tested the estradiol-linked-nor-nitrogen mustard, estramustine (EM), against glioblastoma cell cultures obtained from operative specimens. EM is known to exert its antiproliferative action in prostatic cancer cells by binding to microtubules and promoting disassembly. This action is not related to the steroid or alkylating moiety and seems to be a result of the carbamate-ester bond between the two. We synthesized estrone analogs of EM without an alkylating moiety and with structural similarities to the carbamate-ester portion of EM. The non-alkylating estrone analogs inhibited as much as 90% of DNA synthesis, measured by ^{3}[H]-thymidine incorporation assays, at a concentration of 10^{-5}M. One agent, JE208, inhibited DNA synthesis nearly as much as EM. This agent also resulted in morphologic changes in glioblastoma cells similar to that of EM. The second objective of this report was to establish an innovative combination chemotherapeutic regime containing EM to further exploit its antiglioma actions. We tested EM *in vitro* in combination with the protein kinase C inhibitor and estrogen antagonist tamoxifen (TAM) and the antitumor antibiotic bleomycin (BLM). TAM was used since PKC is involved in microtubule function and since EM may exert estrogenic side-effects in patients. The combination of EM and TAM was more potent then either agent alone in inhibiting as much as 98% of DNA synthesis of glioblastoma cells. BLM has been reported to be more cytotoxic to cells in G2/M phase of the cell cycle. We first demonstrated that EM causes the accumulation of glioblastoma cells in G2/M, presumably due to its antimicrotubule action during metaphase. Then we showed enhanced cytotoxicity of BLM when cells were pretreated with EM. This report offers preclinical data on the use of EM and non-alkylating synthetic estrone analogs in the pharmacologic treatment of malignant glioma. Since combination chemotherapy has many advantages over single agent therapy, we also presented two *in vitro* combination models for exploiting the unique characteristics of estramustine.

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INTRODUCTION

Primary tumors of the brain remain without effective therapy

During the past decade, there have been significant achievements in the modern treatment of cancer. However, brain tumors have remained an elusive challenge to cancer researchers around the world. Primary tumors of the brain now afflict approximately 12,000-17,000 people annually many of whom are still in a productive stage of life. It is striking that the number of patients dying each year from brain tumors exceeds 10,000 resulting in a high case:fatality ratio (Salcman, 1980). Although the incidence of brain neoplasms comprises less than 2% of all cancers (Katakura and Yoshimoto, 1988), it makes up a much larger portion of total cancer deaths. The proportion of expected life lost due to tumors of glial origin has been recently estimated to be 76% (Kallio *et al.*, 1991). Even more disturbing are recent reports which demonstrate an increase in the incidence of malignant brain tumors in populations world-wide (Ben-Schlomo and Davey Smith, 1989; Greig *et al.*, 1990; Kallio *et al.*, 1991, Mao *et al.*, 1991). It has been argued that this increase cannot be explained simply by the introduction of improved diagnostic methods (Sant *et al.*, 1988).

Of the primary tumors of the brain, those of glial origin comprise approximately 50-60% (Youmans, 1990). These include the astrocytoma, oligodendroglioma, ependymoma, and medulloblastoma. The most common glial derived tumor, however, is the glioblastoma multiforme comprising over half of these neoplasms. The glioblastoma is a highly malignant tumor occurring most often after the fifth decade. It is believed to arise from the malignant transformation of less malignant astrocytic tumors following the continuum: astrocytoma, anaplastic astrocytoma (fibrillary), glioblastoma multiforme. Some glioblastomas may also arise *de novo*. The typical microscopic appearance is characterized by coagulative necrosis with surrounding pseudopalisading, cellular and nuclear atypia, and capillary endothelial proliferation (Harsh and Wilson, 1990). These tumors are highly aggressive and rapidly invade normal brain parenchyma making total surgical resection virtually impossible.

The prognosis for patients with glioblastoma is dismal. The most important prognostic factor for survival is age, however even younger patients rarely survive two years. A recent report estimates the probability of survival to one year to be 31% in those over age 50 years (Sant, 1988). Salcman (1980) obtained a median survival after operation of six months with a two-year survival rate of 7.4%. Current treatment for this disease is limited and usually involves corticosteroids to reduce intracranial pressure, operation for maximal reduction of tumor mass (see Ransohoff, 1976; Ransohoff *et al.* ,1986) radiation (see Nelson *et al.*, 1986), and chemotherapy (see Brandes *et al.* 1991). The most widely used chemotherapeutic agent is bis-chloro-nitroso-urea (BCNU) which has had moderate success in improving survival (Salcman, 1990). However, these data provide no major break-throughs and no dramatic alterations in the course of the disease have been observed over the past thirty years.

Do sex hormones influence the growth of brain tumors?

That sex steroids may influence the growth of human brain neoplasms was first reported by Cushing and Eisenhardt (1929) who observed the rapid progression of a meningioma in a patient during pregnancy. This finding was later confirmed in a larger study (Bickerstaff, Small, and Guest, 1958). More recently, a number of clinical and basic studies have provided evidence that suggests sex hormones may promote the proliferation of tumors of the central nervous system. First, women with breast cancer, a neoplasm commonly influenced by estrogens and progesterone, have been shown to be at increased risk of meningioma (Schoenberg, Christine, and Whisnant, 1975). Second, many intracranial neoplasms display increased incidence in relation to the sex of the patient: meningiomas are preponderant in woman, while gliomas are more common in men (Zulch, 1965). Third, in an experimental animal model, it was shown that the onset of benzpyrene-induced gliomas in rats was delayed by castration and ovariectomy (Hopewell, 1975). Also, there has been a recent study suggesting an association between brain tumors and menopausal status

(Schlehofer, Blettner, Wahrendorf, 1992). Schoenberg, Christine, and Whisnant (1975) found a two fold increase in the expected incidence of meningiomas in patients with breast cancer. Finally, at least in normal tissue, steroids induced neural cell growth in vitro by acting on glial cells (Vernadakis, Culver, and Nidess, 1977).

Sex hormone receptors have been identified in a variety of human tumors (Stedman, Moore, and Morgan, 1980). Several investigators have measured steroid receptors in central nervous system neoplasms. Estrogen receptors were found in four of six meningiomas (Donnell, Meyer, and Donegan, 1979); and in a larger study estradiol binding was detected in meningiomas, schwannomas, and neurofibromas (Martuza, MacLaughlin, and Ojemann, 1981). The latter investigation also revealed the presence of progestin receptors in a small number of meningiomas. Since then other studies have confirmed the presence of estrogen, androgen, and progestin receptors in human meningiomas (Tilzer *et al.*, 1982; Cahill *et al.*, 1984; Kornblum *et al.*, 1988).

Estradiol and progestin receptors were also found in tumors of the spinal cord (Concolino *et al.*, 1984). The tumors examined included ependymomas and astrocytomas. Acoustic neuromas may contain estrogen receptors as demonstrated by immunohistochemistry (Kasantikul and Brown, 1981).

Many of these studies, especially those with neuroepithelial tumors, are difficult to interpret due to the small number of specimens examined. Furthermore, a finding common to most of the studies is the heterogeneity of the sex steroid receptor status of the tumors. Not all tumors contain steroid receptors, and of those that do, the specific receptor type in greatest quantity differs even between tumors of similar histology. For example, Poisson *et al.* (1983) reported eight of nine gliomas androgen receptor positive, two neoplasms also had estrogen receptors and two had progesterone receptors. Conversely, Kornblum *et al.* (1988) found no steroid receptors in any of the eleven gliomas they examined. Fujimoto *et al.* (1984) were unable to detect the presence of estrogen receptors in twelve glioblastomas, two cerebellar astrocytomas, and two ependymomas. They did note estrogen receptor positivity

in all three medulloblastomas studied. Neither progesterone nor estrogen receptors were identified in gliomas in a study of 30 intracranial tumors (Courriere *et al.*, 1985). Interestingly, this study found progesterone receptors in all twelve meningiomas tested but not one of these tumors contained estrogen receptor proteins. Progesterone, estrogen, androgen, and glucocorticoid receptor levels were measured in another study of 12 gliomas (Brentani *et al.*, 1984). Positive levels (greater than 10 fmol/mg protein) of progesterone receptor were identified in 50%, estrogen receptor in 17%, androgen receptor in 42%, and glucocorticoid receptor in 58%. A more recent report noted the presence of progestin receptors in 3 of 21 glioblastomas, androgen receptors in 7 of 21, and no estrogen receptors in any of 21 glioblastomas (Stojkovic *et al.*, 1990). Finally, Paoletti *et al.* (1990) demonstrated in a large study of neuroepithelial tumors 39% of tumors with glucocorticoid receptor positivity, 22% with androgen receptors, 9% with estrogen receptors and only 4% with progesterone receptors. Again, these studies are difficult to interpret because the methods for determining receptor positivity as well as the definition of positivity are not uniform.

In vitro studies have shown that estrogen and progesterone stimulate the growth of meningiomas in culture, and that this estrogen-induced growth can be inhibited by progesterone and, to a lesser extent, the anti-estrogen tamoxifen (Jay *et al.*, 1985). In contrast, it has also been shown that progesterone enhances growth of meningioma when measured by a tumor stem cell clonogenic assay (Grunberg *et al.*, 1987). In addition, the progesterone receptor antagonist RU 486 inhibits the growth of meningioma cells *in vitro* (Olson *et al.*, 1986) and in nude mice (Olson *et al.*, 1987). One study examined the effect of host sex on the growth of a human glioblastoma heterotransplanted into nude mice (Verzat *et al.*, 1990). The tumors implanted into male nude mice demonstrated enhanced growth rate compared to the same tumors when implanted into female nude mice. The authors postulated that this difference was a result of the contrasting hormonal environment. However, studies

more closely examining the role of sex steroids in the proliferation of glioblastoma cells have not been undertaken to date.

The importance of this work is indicated by the poor prognosis of patients with glioblastomas and other gliomas. Effective medical therapy is lacking especially when the tumor remains unresectable (Suzuki, 1988). The current knowledge of steroid receptors and hormone therapy in patients with carcinoma of the endometrium, prostate, and, especially the breast remains far more advanced than our understanding of the relationship of hormones to brain neoplasms (Menon *et al.*, 1977; Patterson and Battersby, 1980; Henderson *et al.*, 1982; Leung, 1982; Hollander, 1985). Perhaps an increased understanding of this relationship will provide new methods of extending and enhancing the quality of life of patients with glial cell tumors.

Estramustine: an estrogen mustard

The discovery of estrogen receptors in human neoplasms provided researchers with a potential chemotherapeutic target. The use of antiestrogens in estrogen receptor–positive breast cancer is well documented (Jordan, 1986). However, perhaps a more innovative therapy was first realized in the early 1970's with the development of steroid based alkylating agents (Konveys and Hogberg 1974). The rationale was to specifically target cytotoxic alkylating agents to hormone dependent neoplastic cells (Leclerq, Deboel, and Heuson, 1976; Leclerqc, Heuson, and Deboel, 1976). Many of these compounds were similar in that the alkylating moiety was conjugated to the steroid A–ring at position 3 (Catsoulacos and Boutis, 1973; Leclercq and Heuson, 1978; Catsoulacos, Politis, and Wampler, 1979). The compound estramustine (EM) is a 17β–estradiol derivative conjugated with a nor–nitrogen mustard through a carbamate–ester bond on position 3 of the A–ring (Figure 1a). The estrogen was to function as the carrier for the nitrogen mustard to estrogen dependent cancer cells.

The first studies of the cytotoxic effects of estramustine were in breast cancer cells. These early studies in vitro and in DMBA– induced rodent mammary tumors demonstrated promising antiproliferative activity (Muntzing, Jensen, Hogberg, 1979). However, clinical studies using EM in breast cancer patients were less encouraging. Since EM proved effective in inhibiting growth of prostatic cancer cell lines and animal models (Wakisaka, Iwasaki, and Shimazaki, 1979; Hansenson *et al.*, 1988) clinical studies were attempted in patients with advanced prostate cancer (Jonsson and Hogberg, 1971; Mittelman, Shukla, and Murphy, 1976; Nilsson and Jonsson, 1976; Jonsson, Hogberg, and Nilsson, 1977; Nagel and Kolln, 1977; Benson, Gill, Cummings, 1983). These results were more promising. In fact, estramustine is used in Europe, especially in Nordic countries, for treatment of advanced prostatic carcinoma (Walzer, Oswalt, and Soloway, 1984; Johansson, Andersson, and Holmberg, 1991).

Estramustine: a unique mode of action

The mechanism of action of EM was largely believed, at first, to be a result of the selective delivery of the alkylating moiety to cells containing the estrogen receptor. It was later discovered that the cytotoxicity of EM was independent of the alkylating component (Tew, 1983; Hoisaeter, 1984; Tew and Hartley–Asp, 1984). Walker 256 rat mammary cells with acquired resistance to alkylating agents were still susceptible to the cytotoxic actions of estramustine (Tew and Wang, 1982). EM did not appear to induce the characteristic DNA damage, such as cross–linkage and strand break, of the alkylating agents (Tew *et al.*, 1983).

At the same time it was also determined that EM cytotoxicity was not a function of the steroid component. In addition, the major metabolite of estramustine is estromustine (formerly LEO 271) (Figure 1a) and not estradiol (Kadohama *et al.*, 1979; Dixon, Brooks, Gill, 1980; Andersson *et al.*, 1981; Gunnarsson *et al.*, 1981) indicating that cleavage of the carbamate–ester bond is likely not a significant factor in cytotoxicity. DU145 prostatic cancer cells contain no measurable estrogen receptors and are resistant to estradiol treatment yet they

are highly sensitive to growth inhibition by EM (Hartley–Asp and Gunnarsson, 1982). Similarly, while neither Walker 256 or HeLa S3 cells contain estrogen receptors or are inhibited by estradiol, they are susceptible to the cytotoxic actions of EM (Tew 1983).

Therefore, the mechanism of action of EM is independent of the alkylating moiety and the estrogen component of the parent molecule. It is likely that the carbamate–ester bond plays a significant role in mediating the cytotoxic effects of estrogen mustard. Clues to the cellular and molecular target of EM and its carbamate ester bond were uncovered in the early 1980's. When exposed to cytotoxic concentrations of EM, DU145 cells were found to exhibit mitotic arrest specifically while in the metaphase (Hartley–Asp, 1983; Tew *et al.*, 1983; Hartley–Asp, 1984; Sheridan, Speicher, and Tew, 1991). It was observed that EM interfered with spindle formation and resulted in a characteristic "rounded–up" appearance of cells. These results were similar to an earlier finding that EM resulted in an increase in the mitotic index of peripheral blood lymphocytes (Evans and O'Riordan, 1975).

Using fish erythropores, a red pigment cell with thousands of radially organized microtubules, as a model, Sterns and Tew (1985) demonstrated that EM in micromolar concentrations disrupts microtubules. A loss of red pigment pulsation was observed when EM caused the disassembly of microtubules from the peripheral margins toward the center. Sterns and Tew also used fluorescein labeled B–tubulin antibody to demonstrate a loss of the normal microtubular array and a rounding up of DU145 cells after exposure to EM for 20 minutes.

EM also inhibited axonal transport in the frog sciatic nerve presumably through the promotion of microtubule disassembly (Kanje *et al.*, 1985). It was proposed that EM was able to act in a "detergent–like manner" and disrupt microtubules by interacting with microtubule associated proteins (MAPs) (Sterns and Tew, 1985). Indeed, it was soon shown that certain MAPs bound EM in vitro (Wallin, Deinum, and Friden, 1985). This differs from the mechanism of taxol which stabilizes microtubules and enhances assembly (Schiff, Fant, and Horwitz, 1979) and from the vinca alkaloids which inhibit microtubule

assembly by preventing tubulin addition to the ends of the mitotic spindle (Jordan, Thrower, and Wilson, 1991).

MAPs are specialized proteins associated non–covalently with microtubules. They can interact with microtubules to promote microtubule assembly. It has been suggested that MAPs may allow for functional specialization of microtubules (Vallee *et al.*, 1986). Indeed, some MAPs are tissue and cell specific (Bloom *et al.*, 1986, Binder, Frankfurter and Rebhun, 1986). MAP 2 is almost exclusively found in neuronal tissue or neural crest derivatives (Caceres *et al.*, 1984; Stearns and Binder, 1987) and MAP–1 is found in both neuronal and non-neuronal cells (Bloom, Schoenfeld, Vallee, 1984). When it was demonstrated that EM bound both MAP–1 (Stearns *et al.*, 1988) and MAP–2 (Stearns and Tew, 1988) and resulted in not only the inhibition of microtubule assembly but the disruption of intact microtubules it was clear that this drug was a novel anti–microtubule agent.

Estramustine binding protein accumulates estramustine in cells

Estramustine was widely studied using prostatic cancer cells largely because of early work which demonstrated uptake and retention of EM *in vivo* by the rat prostate (Plym Forshell and Nilsson, 1974; Hoisaeter, 1976; Appelgren *et al.*, 1978; Kruse, and Hartley-Asp, 1988). A 40-50kD high affinity rat estramustine binding protein (REMBP) was later discovered by gel chromatograpy in the cytosol of ventral prostate homogenate (Forsgren, *et al.*, 1977; Forsgren *et al.*, 1979). This protein was distinct from the steroid receptors and natural steroids were poor competitors for binding sites when compared to estramustine and estromustine. In fact, it seems as if REMBP was previously discovered independently by other groups and described as a prostatic secretory protein called prostatein (Lea, Petrusz, and French, 1979) and prostatic binding protein (Heyns, 1977; Heyns *et al.*, 1978). There may even be an increase in EMBP in neoplastic cells since treatment with radio-labeled EM resulted in the measurement of three times as much radioactivity in prostate cell cytosol from

rats with prostatic cancer than in those with benign prostatic hyperplasia BPH (Forsgren and Bjork, 1984).

The human EMBP (HEMBP) was soon characterized as a 54 kD glycoprotein by gel filtration and HPLC with a sedementation coefficient of 3.6S (Bjork *et al.*, 1982). It was suggested that HEMBP might serve as a prognostic indicator of response to EM therapy in prostatic cancer. Also, EMBP levels may be regulated by steroid hormone levels. REMBP decreases after castration but is restored to previous levels following replacement of androgens (Pousette *et al.*, 1981).

Using immunohistochemistry, REMBP immunoreactivity was found in cultured human glioma cells sensitive to the anti-proliferative effect of estramustine (von Schoultz *et al.*, 1988). This finding not only has major therapeutic implications but also is important on a more fundamental scientific level. EMBP has recently been discovered in human brain tumor specimens using REMBP polyclonal antiserum with cross reactivity to HEMBP in a radioimmunoassay (von Schoultz *et al.*, 1991). Both astrocytoma and meningioma tissue samples contained levels higher than in epileptic brain and brain specimens taken at autopsy. If EMBP production is increased in human brain tumor compared to surrounding brain, potential selective chemotherapy using EM would be possible.

Estramustine as an antiglioma agent

The finding that MAPs are abundant in glial cells and gliomas (Couchie *et al.*, 1985; Couchie, Charriere–Bertrand, and Nunez, 1988), that level of differentiation and quantity of microtubules are inversely related in neoplastic astrocytes, and the finding of EMBP immunoreactivity in human glioma cells led to the possibility of developing EM for the treatment of glioma. Anti–microtubule therapy in other refractory cancers has proven efficacious (McGuire *et al.*, 1989) and it was hoped that the microtubule, MAPs, and EMBP might provide a potent and specific target for the treatment of malignant glioma.

Indeed, EM was shown to inhibit the growth of cultured malignant glioma cells (von Schoultz *et al.*, 1988). Glioma cell lines U-105MG, U-118MG, and U-251MG were inhibited in a dose dependent fashion with concentrations of 1-40 mg/ml after three to six days of incubation. EM is taken up and retained by glioma cells and is readily oxidized to its primary active metabolite estromustine (von Schoultz, Gunnarsson, and Henriksson, 1989). EM inhibition of proliferation was much more pronounced than estradiol or nor-nitrogen mustard alone (von Schoultz, Lundgren, and Henriksson, 1990). These data correlate with that generated from prostatic cancer cells *in vitro* and suggest a similar mode of action for EM in glioma cells. In fact, EM inhibits mitosis and results in G2/M phase cell cycle arrest (*Ibid*). However, the mechanism of EM action in glioma cells has not yet been defined and comparisons to its mode of action in the prostate cell lines are purely speculative. Further work will need to be done to determine if EM binds to MAPs in glioma cells and when HEMBP actually exists in human brain tumors.

In vitro combination chemotherapy

As mentioned previously, current chemotherapeutic options for the management of malignant gliomas are limited and their clinical efficacy is unsatisfactory. No single agent has been able to significantly alter the dismal prognosis of patients with glioblastoma. Combination chemotherapy has many advantages over single agent regimes including less toxicity, greater cell kill, and decreased tumor resistance. We (Piepmeier *et al.*, 1993) and others (von Schoultz *et al.*, 1988, 1990) have shown that antimicrotubule compounds such as estramustine potently inhibit malignant glial cell growth in culture. However, the utility of estramustine *in vivo* may be best realized in a combination chemotherapeutic regime. We selected two agents to evaluate with estramustine in a combination chemotherapeutic model *in vitro*: tamoxifen and bleomycin. What follows is a brief description of the rationale behind this choice of drugs.

Tamoxifen: a protein kinase C inhibitor

The triphenylethylene derivative tamoxifen (TAM) is a member of a class of compounds that are potent inhibitors of protein kinase C (PKC) (O'Brian *et al.*, 1985, 1986). This inhibition is thought to be mediated by direct action at the ATP-binding region of the active site of PKC (O'Brian, Ward and Anderson, 1988). TAM is also an estrogen receptor antagonist used as adjuvant therapy in the treatment breast cancer (Jordan 1986). However, some effects of TAM on breast cancer cells *in vitro* are distinct from its antiestrogenic activity (Reddel, Murphy, and Sutherland, 1983; Sutherland *et al.*, 1983). Indeed, TAM inhibits proliferation in some ER-negative breast cancer cell lines maintained in serum free media (Darbre, Curtis, and King, 1984; Chouvet *et al.*, 1988).

PKC inhibition exerts an antiproliferative effect on cultured gliomas (Pollack *et al.*, 1990a) and may induce differentiation of human glioblastoma cells (Minana *et al.*, 1991). Recently, TAM has been demonstrated to inhibit the proliferation of cultured malignant gliomas acting through a mechanism independent of estrogen receptor blockade (Pollack *et al.*, 1990b). We investigated the effect of combining EM and TAM on the proliferation of cultured human glioblastoma cells since these agents may possess synergistic actions against microtubules. This combination also targets distinct physiologic mechanisms which may enhance the antiproliferative action of either agent when used alone. In addition, some clinical side-effects of EM are estrogen-related, presumably due to increased levels of circulating estrogens and estrogen metabolites (Andersson *et al.*, 1981; Gunnarsson *et al.*, 1981; Daehlin *et al.*, 1986). It is certainly possible that the addition of TAM to an EM regimen may benefit patients suffering from estrogenic side-effects.

Bleomycin is most effective against mitotic cells

Another agent we thought would be useful in combination with estramustine is bleomycin. Bleomycin is a glycopeptide antibiotic isolated from *Streptomyces verticillus* with anti-tumor properties (Umezawa *et al.*, 1966). Its mechanism of action is by DNA

damage through scission likely a result of free radical generation (Twentyman, 1984). In contrast to other anti-cancer agents, bleomycin shows little bone marrow toxicity (Kimura *et al.*, 1972). Pulmonary fibrosis is its major dose limiting side-effect. Bleomycin has found its clinical utility in combination chemotherapy for lymphomas, squamous cell cancers, and testicular carcinoma..

Terasima and Umezawa (1970) first demonstrated the sensitivity of mitotic cells to bleomycin. They syncronized HeLa S3 cells using the mitotic-shake method and found these cells to be most sensitive to bleomycin. Work by Barranco *et al.* (1982) has shown that bleomycin is most cytotoxic to cells in G2/M phase of the cell cycle. Chinese hamster ovary (CHO) cells synchronized in the S-phase with dianhydrogalactitiol and released will show the greatest proportion of cells in G2/M 18 hours later. When CHO cells were exposed to bleomycin at various times after Gal treatment, they were most susceptible to the cytotoxic effect when the greatest number of cells were in G2/M.

Barranco and Humphrey (1971) also showed that when CHO cells were synchronized in various stages of the cell cycle with double thymidine block, they were most sensitive to bleomycin in G2 and M phase. Less than one-tenth the amount of bleomycin was needed to achieve a similar decrease in survival if the cells were in G2 or M as opposed to G1 or S. This approach has also been successful more recently *in vitro* with sodium arsenite as the synchronizing agent (Jan *et al.*, 1990) and in animal studies with ACNU (Shimizu *et al.*, 1980). Indeed, in humans the effectiveness of bleomycin in combination chemotherapy regimes may be related to a cell kinetic mechanism.

Bleomycin has demonstrated antitumor activity and increased survival in a rat brain tumor model (Morantz *et al.*, 1983). An *in vitro* study used bleomycin at a concentration of 10 mU/ml and inhibited the growth of 7 of 15 cultured gliomas (Bogdahn 1983). Clinical trials with bleomycin in the treatment of brain tumors have produced mixed results (Takenchi 1975; Feun *et al.*, 1991).

We have, therefore, set out to increase the susceptibility of glioblastoma cells to bleomycin by pretreating cells with estramustine. We first sought to demonstrate a significant accumulation of cells in G2/M with estramustine. Then, we established a dose response antiproliferative action of bleomycin on our cells. Finally, we examined the effect of bleomycin on cells pretreated with estramustine.

Structural analogs of estrone without an alkylating moiety

Since the antimicrotubule effect of EM is observed only when the compound is intact and not through its alkylating or steroid constituents, we postulated that the structural relationship of the carbamate-ester bond to position 3 of the A-ring plays a role in its antimicrotubule action. This is consistent with the structure of other microtubule inhibitors such as taxol (McGuire *et al.*, 1989) and nocodazole (Gupta 1986) which contain a carbamate group at either the 2 or 3 position of a heterocyclic or homocyclic A ring. To address whether structural variants of estrone without an alkylating moiety could inhibit the proliferation of gliomas in culture in a similar fashion as EM, we synthesized estrone analogs with non-alkylating carbamate structures on the A ring. Each of these analogs were tested in vitro for inhibitory action against glioblastoma cells to assess the relationship between structure and antiproliferative efficacy.

Summary of Aims

The aims of this thesis are many fold. Since the literature about steroid receptors in gliomas is confusing we examined operative specimens of glioblastoma for the presence of androgen, estrogen, and progesterone receptors in an attempt to clarify this data. We then sought to determine the effect of various sex steroids on the proliferation of cultured glioblastoma cells. These cells were discovered to be estrogen unresponsive, and we subsequently examined the effect of estramustine on glioblastoma cells in culture in order to further clarify its antiglioma action. Next, we synthesized analogs of estrone structurally

similar to estramustine but without the alkylating moiety and tested them for antiproliferative activity against glioma cells. This was done in an attempt to demonstrate that estramustine action is not a function of the alkylating moiety and to possibly determine the optimal drug structure for microtubule inhibition.

Then we turned our attention to the possibility of combining estramustine with other agents. After finding an inhibitory effect of the estrogen antagonist tamoxifen on the growth of the cells we further examined the inhibitory response of our cultures to this drug. We then sought to increase the efficacy of tamoxifen therapy by pretreating cells with estramustine. Finally, we wished to confirm the ability of EM to arrest cells in G2/M and then attempt to enhance the cytotoxic effect of bleomycin by estramustine pretreatment.

GENERAL METHODS

Chemicals

EM was a gift from Pharmacia Inc., Helsingborg, Sweden and was prepared as a 1000-fold stock in dimethyl sulfoxide (DMSO). Tamoxifen (Sigma Chemicals) was prepared as a 1000-fold stock solution in 95% ethanol. Bleomycin sulfate (Blenoxane) was generously supplied by Dr. T. Dugan at the Oncology Division of Bristol-Meyers Squibb Co., Evansville, Illinois, and was prepared as 1000-fold stock concentrations in sterile water.

Estradiol, dihydrotestosterone, R5020, R1881, and progesterone were obtained from Sigma chemicals and prepared as 1000-fold stock concentrations in 95% ethanol. All radiolabelled steroids were purchased from New England Nuclear (Boston) and stored diluted in either benzene:ethanol (9:1) or toluene:methanol (9:1) at -20 ^{o}C in the dark and routinely tested for purity one week before use by thin-layer chromatography according to manufacturer's recommendations.

Drug synthesis

Analogs of estrone (Figure 1b) were synthesized by Jan Zielinski of the Department of Obstetrics and Gynecology. NMR spectra were recorded on a Bruker MW-500 spectrometer in DMSO-d_6. Mass spectral data were obtained on a Kratos Ms-80 RFA spectrometer at 70 eV. The starting materials, 2-amino- and 4-aminoestrone were synthesized according to the procedure described by Stubeunrauch and Knuppen (1976)

N-Acetyl-2-aminoestrone **(JE213)** was prepared from 2-aminoestrone by reacting with acetic anhydride in methanol: MS- m/z (relative intensity), 327 ($M^{+\cdot}$, 42), 309 (8), 285 (100); ^{1}H-NMR- ∂ 0.826 (s, 3H, C_{13}-CH_3), 2.064 (s, 3H, CH_3-CO-), 6.543 (s, 1H, C_4-H), 7.479 (s, 1H, C_1-H), 9.301 (s, 1H, C_3-OH), 9.427 (s, 1H, -NH-)

Reaction of 2-aminoestrone with an equimolar amount of ethyl chloroformate in N,N-dimethylformamide (DMF) in the presence of sodium bicarbonate produced ethyl carbamate

(JE208): MS- m/z (relative intensity), 357 ($M^{+\cdot}$, 100), 311 (61), 285 (21), 284 (22), 270 (5); ^{1}H-NMR- ∂ 0.826 (s, 3H, C_{13}-CH_3), 1.213 (t, 3H, J=7.0 Hz, $\underline{CH_3}$-CH_2-O-CO-), 4.078 (q, 2H, J=7.0 Hz, CH_3-$\underline{CH_2}$-O-CO-), 6.522 (s, 1H, C_4-H), 7.426 (broad, 1H, C_3-OH), 8.061 (s, 1H, C_1-H), 9.288 (broad, 1H, -NH-)

Ethyl carbamate **(JE205)** was prepared in the same manner: MS- m/z (relative intensity), 357 ($M^{+\cdot}$, 100), 311 (47), 285 (29), 284 (31), 270 (40); ^{1}H-NMR- ∂ 0.823 (s,3H, C_{13}-CH_3), 1.213 (broad, 3H, $\underline{CH_3}$ -CH_2-O-CO-), 4.027 (broad, 2H, CH_3 -$\underline{CH_2}$-O-CO-), 6.651 (d, 1H, J=8.4 Hz, C_2-H), 7.020 (d, 1H, J=8.4 Hz, C_1-H), 8.098 (broad, 1H, -NH-), 8.942 (s, 1H, C_3-OH).

Cyclization of carbamate (JE208) in DMF at 150^{o}C yielded 2,3-oxazolonylestra-1,3,5(10)-triene-17-one **(JE212)**: MS- m/z (relative intensity), 311 ($M^{+\cdot}$, 100), 255 (17), 254 (19); ^{1}H-NMR-∂ 0.833 (s, 3H, C_{13}-CH_3), 6.937, (s, 1H, C_4-H), 6.978 (s, 1H, C_1-H), 11,383 (broad, 1H, -NH-)

Cell culture

Tumor cultures of glioblastoma multiforme were obtained from operative specimens (Table 1). Those which were assayed for steroid receptor proteins were immediately frozen in liquid nitrogen following surgical removal. Confirmation of histological diagnosis was provided by neuropathology. Pieces of tumor tissue were washed in PBS and then mechanically minced and dissociated with trypsin. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal calf serum (Sigma), penicillin 100 units/ml, and streptomycin 0.01 mg/ml, at a temperature of 37^o C in an atmosphere of 5% CO_2. Cells from monolayers were lifted with 0.25% trypsin in 5 mM EDTA and passed at a dilution of 1:10. The human glioblastoma cell line HS683 (American Tissue Culture Collection Rockville, Md.) was cultured in a similar manner.

One of the tumor cultures, J889H, was selected most often for study because it grew rapidly, maintained a consistent morphology following multiple passages and stained for glial

fibrillary acid protein by immunohistochemistry. J889H was obtained from a parietal lobe glioblastoma in a 58 year old man who had been treated with radiation therapy and Lomustine (CCNU) prior to obtaining this sample. Surgery was performed for recurrence of this glioblastoma following this therapy. Karyotyping of J889H revealed an aneuploid tumor with a chromosome count of 70. This tumor contained numerous abnormal chromosomes with reorganizations, deletions, and translocations consistent with a highly malignant glioma. Our laboratory has extensive experience with this tumor culture.

Cell preparation

All proliferation studies were performed as described below. Cells were lifted with 0.25% trypsin in PBS with 5 mM EDTA, counted in a hemocytometer, and plated at a density of 2 x 10^4 cells/cm^2/100µl in 96-well microtiter plates. The cells were incubated and allowed to attach for 24h, and the plates were examined to assure an equal distribution of cells. The media was then withdrawn and 100 µl of experimental media was added containing the appropriate drug concentration.

Control samples were used in each experiment and received medium containing the same concentration of DMSO and/or ethanol as the drug-treated cells (0.1%). All experiments were performed at least in quadruplicate but most often in sextuplicate. Estramustine, bleomycin, tamoxifen, estradiol, progesterone, DHT, estrone analogs, or combinations were diluted to the appropriate concentrations in medium and added in 100ml volumes.

^{3}H-Thymidine incorporation

After incubating for the required time of the experiment, 1µCi ^{3}H-thymidine was added to each well in 25 ml volumes. After an additional 4h incubation, the cells were lifted with a PHD cell harvester (Cambridge Technology, Inc.), transferred to glass fiber strips

which were then placed in scintillation vials with 2 ml scintillation fluid, and thymidine incorporation was measured with a Beckman LS 2800 liquid scintillation counter.

Flow cytometry

Cells were plated at a density of 2 x 10^4 cells/cm^2 in large 150 cm^2 flasks in order to obtain at least 1 X 10^6 cells for analysis. The cells were allowed to attach overnight and were then treated with drug in a volume of 23 ml media for the specified number of hours. At the conclusion of incubation, the cells in each sample were then washed in PBS, lifted with 0.25% trypsin in EDTA, and resuspended in 2 ml of ice-cold PBS. The cells were fixed by three stepwise additions of 2 ml each of ice-cold 95% ethanol. A minimum of one hour after fixation, the cells were resuspended in 1 mg/ml RNAse (Sigma) for 30 minutes at 37°C and then stained with 0.05 mg/ml propidium iodide (Sigma) for one hour on ice.

Flow cytometric analysis was performed with a FACS IV flow cytometer (Bectom-Dickinson, Mountain View, CA) by Rocco Carbone at the Yale Comprehensive Cancer Center, New Haven, CT. The cells were excited at 488nm, and the emission was collected above 590 nm. A minimum of 20,000 cells were analyzed for each sample. Cell cycle analysis was performed according to the mathematical model of Jett (1978).

Cytosol estrogen, progesterone, and androgen receptor assays

Samples from operative specimens were frozen in liquid nitrogen immediately upon removal in the operating room The tissue was then stored at -80°C for up to 10 months. The following procedures were all performed on ice. The tissue was weighed and then homogenized in ice-cold 10mM Tris ((Tris (hydroxymethyl) aminomethane) hydrochloride buffer containing 1.5 mM disodium EDTA, 1 mM dithiothreitol, 10 mM sodium molybdate, and 10% glycerol, pH 7.4 (TEGDMo buffer). Tissues were homogenized in a motor-driven glass Teflon homogenizer in 10 vols. of TEGDMo buffer. The homogenates were centrifuged at 45,000 rpm (100,000 x g) using a Ti70.1 rotor (Beckman) in a OTD-75

DuPont-Sorvall ultracentrifuge refrigerated to 1°C. The supernatents (cytosol fraction) were withdrawn with pre-chilled Pasteur pipettes and used immediately.

For saturation binding analyses, samples were incubated with a range of concentrations of the appropriate ^{3}H-labeled ligand. In the case of the cytosol estrogen receptor (ER) assays, incubations were performed with ^{3}H-estradiol (2nM) at 0-4 °C for 20-24 h. The labeled steroid was added dissolved in 100 ml TEGDMo buffer. Parallel incubations, used in correcting for non-specific binding, contained 50-fold molar excess of unlabelled diethylstilbesterol (DES) in addition to labeled estradiol. Duplicate 100 µl samples of each incubate were then applied to columns made from 1.5 ml pipet tips. The columns contained Sephadex LH-20 equilibrated with TEGDMo buffer at 0-4 °C. Each sample was washed into the column with 100 ml of the same buffer. Flow through the columns was arrested for 30 min to reduce non-specific binding by differential dissociation. At the end of this time the remaining ^{3}H-steroid was eluted with 400 µl directly into scintillation vials. Five ml of Optifluor scintillation fluid were added to each sample which was then assessed for radioactivity in a Beckman β-counter.

Androgen receptors (AR) were assayed in a similar manner. Aliquots of cytosol were incubated with 12 nM ^{3}H-R1881 (an androgen receptor agonist) and 6.6 mM triamcinolone acetomide (added to block corticosterone and progestin receptor binding) for 20-24 h at 0-4 °C. Parallel incubations to correct for non-specific binding were performed with 100-fold molar excess of unlabelled R1881. The remainder of the assay was performed as above.

Progesterone receptors (PR) were also assayed in a similar manner. Aliquots of cytosol were incubated with 2.4 nM ^{3}H-R5020 (a progestin agonist) and 1 mM dexamethasone (added to block glucocorticoid binding) for 20-24 h at 0-4 °C. Parallel incubations with 60-fold molar excess of unlabelled R5020. The remainder of the assay was performed as in the estradiol receptor assay.

The cytosol protein content was assayed using the well known method of Bradford (1976) with bovine serum albumin as standard.

Data analysis

The results of the 3H-thymidine incorporation analyses were analyzed by averaging the CPMs of the experimental groups and comparing them using the Student's T-Test on computer software (Stat Works). Dose response curves were analyzed using one-way analysis of variance (ANOVA) on the computer software StatView +.

RESULTS

Steroid receptor analysis in operative glioma specimens

This experiment examined tissue from operative specimens of glioma for the presence of steroid receptor proteins. The specimens studied included H1289G a glioblastoma from a woman; WG, IH, and AM glioblastomas removed from men, and JMz, an anaplastic oligodendroglioma. The specimens were assayed for the presence of androgen, estrogen, and progesterone receptors. Freshly dissected rat uterus was used as a positive control.

The specimens examined displayed positive, but very low levels of receptor binding for all hormones. However, not every specimen was positive for every receptor. Using 10 fmol receptor/mg cytosol protein as the lower limit of positivity, 3 of 5 tumor specimens contained androgen receptors, 3 of 5 estrogen receptors, and 3 of 5 progesterone receptors (Table 2). The greatest level of receptor was 74 fmol/mg (ER in tumor H1289G) and the lowest positive value was 12 fmol/mg (PR in tumor IH). This is compared to greater than 1000 fmol/mg ER in the rat uterus. Only one tumor contained all three receptors (AM), and two tumors contained only one receptor type (ER-JMz and AR-WG).

Response of cultured glioma cells to steroid hormones

These studies focused on the response of cultured gliomas to various hormones. Primary cell cultures of the following tumors were studied: AM, H1289G, SZ, AMe, JML, and SC. Mature astrocytes from a temporal lobectomy for epilepsy were also studied. The methods are outlined above with the following distinctions. Estrogen, progesterone, and DHT were added to the culture media at a concentration of 10^{-7}M with 0.1% EtOH serving as control. The media differed in that 10% gelded horse serum (Lowell Crowther Ranch, Sanford, CO) was used instead of fetal calf serum since the horse serum was mostly steroid free. Cells were incubated in the steroid containing media in quadruplicate microtiter wells for 72 hours before ^{3}H-thymidine labeling took place. The experiment was repeated twice to confirm the data.

The results of these studies were unimpressive. None of the steroids tested had any stimulatory or inhibitory effect on any of the cell cultures in the concentration or duration studied when compared to control. Figure 2 shows the results for the AM cells, but these results were identical to those of the other gliomas. The steroid concentration was increased to 10^{-6}M, but no difference was seen (Figure 3). The fetal astrocytes also did not respond to the sex hormones (data not shown).

Antiproliferative effect of estramustine in malignant glioma cell cultures

Having failed to demonstrate any effect of steroid hormones on glioma cells and with receptor assay results difficult to interpret we decided to study the estrogen mustard in our cell systems since its reported actions are not mediated through an estrogen receptor.

We studied the cell cultures J889H, RB, JMl, and AM. We used 1.0, 2.5, and 5.0 µg/ml EM ($2.27x10^{-6}$M, $5.68x10^{-6}$M, $1.14x10^{-5}$M) and found a dose response inhibitory curve at these concentrations during 24h of exposure (Figure 4-5). EM at the highest concentration significantly inhibited cell proliferation from 60 to 82% of control depending on the cell line tested ($p<0.02$, Student's T-Test). This inhibition of thymidine uptake was dependent on dose in all tumor cultures ($p<0.001$, ANOVA). EM at 2.5 µg/ml ($5.68x10^{-6}$ M) inhibited about 50% of cellular proliferation in all cultures. All experiments were performed in sextuplicate microtiter wells.

As mentioned in the General Methods, J889H was often selected for further testing due to its high susceptibility to EM and that it has been very well characterized in our lab. We decreased the concentration of EM for the next dose response study. J889H was exposed, as usual in sextuplicate microtiter wells, for 24h to 0.2, 1.0, and 2.5 µg/ml EM ($4.54x10^{-7}$M, $2.27x10^{-6}$M, $5.68x10^{-6}$M). EM inhibited proliferation in a dose dependent fashion ($p<0.001$, ANOVA). Even at the lowest dose EM significantly inhibited DNA synthesis measured by thymidine uptake (Figure 6).

Antiproliferative action of non-alkylating estrone analogs of estramustine on glioma cells

Next, we wanted to observe whether the alkylating portion of EM was a requirement for DNA synthesis inhibition, or whether our synthesized estrone analogs with a similar non-alkylating structure on the A ring (Figure 1b) could similarly inhibit DNA synthesis. Since the molecular weights of these agents varied we decided to use 10^{-5}M concentrations since this most closely approximated 5mg/ml EM. For these experiments J889H was the tumor culture used.

After 24h of exposure to EM and the 4 synthesized estrone analogs, J889H was inhibited by all compounds but in various degrees (Figure 7). EM was clearly the most effective agent reducing thymidine incorporation to 5% of control. JE 208 was the most effective analog, inhibiting DNA synthesis by 91% of control. The other analogs, JE 205, JE 212, and JE 213 reduced thymidine incorporation by 68%, 90%, and 58%, respectively. The above experimental groups were represented in sextuplicate.

To assess the rapidity of the antiproliferative action, we applied the drug to the tumor cultures and then immediately incubated with labeled thymidine so that the cells were exposed for only 4h to drug. Even after only 4h of exposure these agents inhibited DNA synthesis to a lesser degree but in a similar fashion with EM being most effective followed by JE 208, JE212, JE205, and JE213 (Figure 8). Again, the experimental groups were represented in sextuplicate.

Effect of tamoxifen on glioma cell proliferation

We next turned toward the use of tamoxifen in our cultures for two reasons. First we wanted to block any possible estrogen receptor effect of estramustine to further clarify its mechanism of action as ER independent. Also, we wanted to reproduce tamoxifen inhibition of malignant glial cell growth reported by one other lab (Pollack et al., 1991), and then determine if we could enhance its antiglioma effect by combination with estramustine.

We first used concentrations of tamoxifen of 0.2 μg/ml and 5.0 μg/ml for 48h. These concentrations were the minimum and maximum concentrations used previously by Pollack *et al.* (1990a). We studied cultures of J889H, H1289G, RB, and SZ. Figure 9 shows the rather narrow range of tamoxifen inhibition of DNA synthesis. Next, we shortened the duration of exposure to 24h and expanded the dosages to 0.2, 1.0, 2.5, and 5.0 μg/ml. Figure 10 shows the dose dependent inhibition of DNA synthesis which is remarkable for the narrow range of toxicity. Next we studied the relationship of DNA synthesis inhibition of tamoxifen to duration of exposure. It was possible that tamoxifen exerted an early effect on DNA synthesis which was transient due to rapid metabolism of the drug. We added varying concentrations of tamoxifen to J889H for 4, 12 and 24 hours. Another group of cells had 3[H]-thymidine added at the time of exposure to tamoxifen to assess the most immediate response. Again, the only significant inhibition of DNA synthesis occurred in the cells exposed to 5.0 μg/ml tamoxifen (Figure 11). There was a time related response to the largest dose of tamoxifen (5.0 μg/ml). However the response to the other concentrations did not improve significantly over time. Interestingly, tamoxifen's inhibitory action on this tumor culture began to be seen at as little as 4h before ^{3}H-thymidine analysis. In all the above experiments the control groups were represented in 12 microtiter wells and the experimental groups in sextuplicate.

Combination chemotherapy in vitro with estramustine and tamoxifen

To assess the effects of the combination of estramustine and tamoxifen on cultured glioblastoma cells we used 2.5 μg/ml EM (5.68×10^{-6}M) and 5.0 μg/ml TAM since these concentrations were previously demonstrated to be effective in inhibiting proliferation. Cells were cultured in sextuplicate for 24h in EM, TAM, or a combination of both and control which contained 0.1% EtOH and 0.1% DMSO. Cell cultures used for this study included J889H, SZ, H1289G, and the commercially available glioblastoma cell line HS683.

TAM and EM independently inhibited DNA synthesis in all three primary cell cultures as well as the commercial cell line. (Figure 12-15). EM significantly inhibited about 67% of DNA synthesis in all cell cultures tested ($p< 0.001$, Student's T-Test). H1289G and SZ were more sensitive to TAM than EM, while HS683 and J889H responded more to EM. The combination of TAM and EM is a more potent DNA synthesis inhibitor than either agent used alone (Table 3). All cultures were inhibited most by this combination. If the concentration of EM is decreased to 1.0 μg/ml ($2.27x10^{-6}M$), this effect is still observed (Figure 16). In this experiment TAM appeared more potent than in the previous study. However, if the concentration of TAM is decreased to 2.5 μg/ml or 1.0 μg/ml it appears as if TAM antagonizes EM (Figure 17).

The characteristic morphological appearance of cells arrested in mitosis is seen in the EM treated cells and in the cells treated with the combination but not in cells treated with TAM alone. (Figure 18a-d). Cells exposed to EM at a concentration of 2.5 μg/ml ($5.68x10^{-6}M$) alone or in combination with TAM display a rounded-up morphology with a retraction of cytoplasmic processes. The cells treated with TAM have a distinct morphology appearing elongated and, at times, bipolar.

Estramustine arrests glioma cells in G2/M phase

In an attempt to evaluate another drug, bleomycin, for efficacy in combination with estramustine, we first sought to determine if EM could increase the fraction of cells in G2/M phase of the cell cycle. As discussed above, this is the phase of the cell cycle during which cells are most sensitive to bleomycin. J889H, H1289G, and HS683 were exposed to estramustine in duplicate 150 cm^2 flasks at a concentration of $10^{-5}M$ for 24h. The cells were then harvested an subjected to flow cytometric analysis. The experiments were replicated three times and the data were pooled. All cells exhibited a large increase in the proportion of cells in G2/M as evident in the DNA histograms (Figure 19a-b). This response was consistent in the cell line and primary tumor cultures. There was as great as a 192% increase

in fraction of cells in G2/M in J889H cells, up to 176% increase in H1289G, and up to 150% increase in HS683 (Table 4).

Decreasing the concentration of EM to 10^{-6}M resulted in a loss of this G2/M accumulation. DNA histograms of control treated cells do not differ from those treated with EM at this concentration (Figure 20). In addition, shortening the time of exposure to 4h even when exposing cells to 10^{-5}M EM, also results in the loss of G2/M accumulation (Figure 21). Interestingly, this shorter duration was previously shown to be sufficient for the inhibition of DNA synthesis by estramustine.

Effect of bleomycin on the proliferation of cultured glioma

In order to select a concentration of bleomycin that would be adequate for our study in combination with estramustine, we established a dose-response curve in J889H. Using the methods for analyzing cell proliferation outlined above, we exposed sextuplicate cell cultures of J889H to increasing concentrations of bleomycin for 24h. This resulted in a marked inhibition of DNA synthesis even at the lowest concentration (Figure 22). We shortened the duration of exposure to 4h to produce a more useful curve for selection of an appropriate concentration of bleomycin (Figure 22). Bleomycin at 1 μg/ml inhibited at least 25% of DNA synthesis and was selected for use in the combination studies.

Combination chemotherapy in vitro with estramustine and bleomycin

Knowing that we could significantly accumulate cells in G2/M with EM making them more susceptible to bleomycin, we then pretreated sextuplicate cell cultures of HS683, J889H, and H1289G with control, 10^{-5}M EM, or 5 X 10^{-6} for 24 hours before 4h exposure to 1 μg/ml bleomycin.

EM (10^{-5}M) alone inhibits up to 70% DNA synthesis while bleomycin results in a 50% decrease in DNA synthesis. However, when cells are pretreated with EM for 24h before exposure to bleomycin, the cytotoxiciy of bleomycin is enhanced (Figure 23-25).

This effect increases with increasing concentration of EM and is consistent in all the glioblastoma cells tested. The combination of the two agents is more cytotoxic than either agent alone, and the effect appears to be additive.

If the percentage increase of cells in G2/M with EM pretreatment is compared with the inhibition of DNA synthesis after exposure to bleomycin we observe that as the percentage of cells in G2/M is increased by EM, the antiproliferative action of bleomycin is similarly enhanced (Figure 26-28).

Early report of estramustine therapy in patients with advanced glioblastoma

With encouraging in vitro data, we have extended our studies with estramustine to clinical trial in patients with glioblastoma who have failed all conventional therapy. All protocols were approved by the human investigations committee. At this time we have experience with three patients. They each received 15mg/kg/d Estracyt (estramustine phosphate) in three divided doses. Two of these patients showed no response and were withdrawn from the study. One patient has been treated for two months and has thus far shown no disease progression. Since we believe the role for estramustine is ultimately in combination chemotherapy, plans to include estramustine in combination with conventional therapy are currently being considered.

DISCUSSION

Summary

This report documents the potential for estramustine as a chemotherapeutic agent against glioblastoma. We demonstrated the effectiveness of EM as an inhibitor of DNA synthesis in a number of glioblastoma cell cultures from operative specimens. It was also shown that synthetic analogs of estrone, structurally similar to EM without an alkylating agent, similarly inhibit glioblastoma cell proliferation. Operative specimens of gliomas contained barely detectable levels of steroid receptor proteins, and no consistent pattern of androgen, estrogen, or progesterone receptor content could be demonstrated among the tumors studied. Therefore, it is unlikely that EM exerts its antiproliferative action via a steroid receptor-related effect. Furthermore, EM and tamoxifen in combination potently inhibit DNA synthesis in all cells tested. If an estrogen receptor was necessary for the antiproliferative effect of EM, we would have expected tamoxifen to reduce rather than enhance the inhibition of DNA synthesis caused by EM. Similarly, the pretreatment of glioblastoma cells with EM enhances the cytotoxic potential of bleomycin alone. This is may be a result of an increase in G2/M phase cells caused by EM. Thus, EM as an antimicrotubule agent is a potent inhibitor of the proliferation of cultured malignant glioma cells, and deserves further study alone and in combination with other agents in patients with glioblastoma.

Sex hormones and tumors

Estrogen, progesterone, androgen, and glucocorticoid receptors have been identified in a variety of human tumors (Stedman *et al.*, 1980; Molteni *et al.*, 1981; Concolino *et al.*, 1984; Hollander, 1985; Meggouh *et al.*, 1991). These include tumors of the head and neck, gastrointestinal tract, liver, pancreas, gallbladder, genitourinary system, various sarcomas, melanoma, and central nervous system in addition to the breast, uterus, ovary, and prostate. The functional and evolutionary significance of this finding remains unclear, however the

direct effect of steroids on DNA implies a possible causal relationship between steroids and cancer. Certainly, it is accepted that sex steroids play a role in the cause, growth, and prognosis of malignancies of the reproductive organs. Whether this is true in tumors of other systems, remains to be demonstrated. The finding of estrogen receptors in malignant tumors of the head and neck and gastrointestinal tract has prompted some investigators to examine the possibility of antiestrogenic control of these tumors (Stedman, 1980; Molteni, 1981). The use of receptor assays to determine prognosis may be another possible extension of this work. However, it is also possible that the finding of hormone receptors in some cancers are merely artifactual and have no therapeutic implications.

That steroid hormones exert influence on the central nervous system has been well established (MacLusky and Clark, 1980; MacLusky and Naftolin, 1981). Steroid hormones can influence the growth of neural tissue (Vernadakis and Timiras, 1967) and also regulate specific glial cell functions (Vernadakis et al, 1978; Gibson and Vernadakis, 1974). Jung-Testas and colleagues (1989) demonstrated *de novo* biosynthesis of steroids in glial cells which supports a hypothesis of steroid influence on the functions of the CNS. Steroid hormones produced by glial cells may direct the organization of CNS neurons during development and may control certain aspects of cellular differentiation of other glial cells. Indeed, it was shown that estradiol can accelerate the maturation of the rodent brain (Heim and Timiras, 1963). These data may indicate that sex hormones can induce tumor formation and growth in the CNS as a result of its ability to affect cellular differentiation. Since steroid hormones exert their influence in cells via specific steroid receptor proteins, it was logical to examine normal and neoplastic glial cells for the presence of hormone receptors.

Most of the literature regarding steroid receptors and brain tumors concentrate on meningiomas which now have been recognized, in many cases, to contain significant levels of progesterone receptor (Grunberg *et al.*, 1991). However, there have been a few reports which investigated the steroid receptor status of gliomas. The largest study by Paoletti and colleagues (1990) analyzed tissue samples from 57 glioma specimens including 25

glioblastomas (GBM). They found 22 tumors contained glucocorticoid receptor (GR), 11 contained androgen receptor (AR), 5 with estrogen receptor (ER), and only 2 with progesterone receptor (PR). Like most investigators they used a dextran-coated charcoal (DCC) method for their receptor assays. The minimal receptor concentration considered positive was 10 fmol/mg protein. The mean concentrations for GR, AR, ER, and PR were only 25.7, 24.1,17.7, 15.9, respectively. Brentani *et al.* (1984) also used a DCC method with a positive receptor level minimum of 10 fmol/mg protein and examined 12 gliomas (6 GBM) for the presence of steroid receptors. They found 2 specimens with ER, 6 with PR, 5 with AR, and 7 with GR. The mean concentration levels were also relatively low at 17, 16, 17, and 12, respectively; barely above the level considered "detectable". Using similar techniques and cut-off values, Fujimoto *et al.* (1984) and Courriere *et al.* (1985) also examined receptor levels in gliomas. The former group examined only ER levels in 20 gliomas, including 12 GBM, and found only 3 contained measurable concentrations with a mean value of 59 fmol/mg protein. The later group examined 2 gliomas, part of a study of a variety of intracranial neoplasms, and found no detectable levels of ER or PR. In 1989, a group from Yugoslavia (Stojkovic *et al.*, 1990) measured sex hormone receptor levels in 29 gliomas (21 GBM) using the DCC method. With concentration levels of 3 fmol/mg protein considered positive for ER and 10 for PR and AR, they discovered 1 tumor with ER, 7 with PR and 9 with AR. These concentrations were no more than 20 fmol/mg protein. . Also using the DCC method Poisson *et al.* (1983) found 8 of 9 gliomas (4 GBM) with measurable AR, 2 with PR, 2 with ER, and 1 with GR. They measured receptor concentration as fmol/gram tumor tissue. This method is not as sensitive since tumor tissue contains variable amounts of protein. Their cut-off level was 100 fmol/g tissue and the concentrations measured were quite high: as much as 1370 fmol/g tissue were noted for ER. Finally, Kornblum *et al.* (1988) found no detectable levels of ER, PR, or AR in any of 11 gliomas (5 GBM) examined. They used 5 fmol/mg protein as the cut-off value for AR and PR, and 10 for ER.

We examined only 5 operative specimens of gliomas and noted results similarly confusing. Three out of five tumors each contained ER, PR, or AR. The concentrations of receptors were slightly higher than in the above studies. This may be a result of the use of Sephadex columns to separate unbound labeled steroid from bound rather than dextran-coated charcoal. Our study demonstrates the heterogeneity of gliomas in regards to steroid receptor protein content. Perhaps only when more specific methods of receptor assay are used, such as Western blotting or immunohistochemistry, will a more definitive conclusion be available. It is likely that gliomas are a heterogeneic tumor with each possessing no characteristic receptor pattern. Whether the presence or absence of steroid receptor proteins correlates with the degree of differentiation remains to be proven. At this time the biologic and therapeutic significance of hormone receptors in gliomas is not clear.

We also have demonstrated the lack of effect of steroid hormones on the proliferation of glioblastoma cells. Unfortunately, only one of the cell cultures (J889H) was studied for the presence of estrogen receptors using immunohistochemistry and determined to possess no ER (data not shown). Only two of the operative specimens studied for receptor content were used in our *in vitro* work. However, it is difficult to compare the tumor at operation to its in vitro counterpart since some of the characteristics of the tumor cells may have been altered by successive passage. Nonetheless, none of the tumor cultures we studied responded to estrogen, progesterone, or androgen at slightly more than physiologic levels. The only other report that studied effects of steroids on cultured gliomas, demonstrated inhibitory effect of dexamethasone on cell growth at concentrations of 10 and 50 μg/ml (supraphysiologic) regardless of GR content and an excitatory effect at a concentration of 0.016 μg/ml only in GR positive cultures (Paoletti *et al.*, 1990). In addition, they also showed a cytotoxic effect of testosterone on cell growth at the supraphysiologic concentrations of 10 and 50 μg/ml but no stimulatory effect at lower concentrations. None of the nine cultures contained androgen receptor protein. Therefore the relationship between receptor presence and cell growth in gliomas remains to be elucidated. Steroids may induce

glioma proliferation via a non-receptor mediated mechanisms or perhaps the steroids induce the dedifferentiation of the glial cell into a cancer and the receptor is present in only the more differentiated tumor. At least in culture it does not appear that the sex steroid hormones enhance the growth of established malignant glioma.

Estramustine is a potent inhibitor of DNA synthesis in glioblastoma cells

This report confirms the results of one other group that EM is a potent inhibitor of human glioma cells. The data produced by von Schoultz and colleagues (1988, 1989, 1990) used concentrations of EM in the range of 5 to 20 μg/ml against human glioma cell lines. Our work is unique in that we have shown that EM is even effective at concentrations as little as one tenth the amount used in previous studies. We also have demonstrated for the first time that EM inhibits the proliferation of primary cell cultures of operative glioma specimens. The effect of EM on glioma cell cycle was also obtained with lower concentrations of EM. It is not clear if the previous group tested EM at lower concentrations or if our cell cultures are more sensitive to EM. However, we have no evidence that would reveal why our cells would have increased sensitivity to EM.

EM effectiveness in glioma may be due to expression of estramustine binding protein (EMBP). von Schoultz *et al.* (1988) demonstrated greater than 90% of 4 different glioma cell lines positive for rat EMBP immunoreactivity. These cell lines were all sensitive to the growth inhibitory effect of EM. However, the use of rat anti-EMBP does not prove the presence of a human EMBP in gliomas. There simply may be cross-reactivity to another cellular protein. Nonetheless, this protein may serve as a prognostic indicator of a positive response to estramustine therapy. Our laboratories are currently engaged in determining whether REMBP is cross-reactive to MAPs in human gliomas.

Analogs of estrone inhibit DNA synthesis in glioblastoma cell cultures

We have produced preliminary data demonstrating the effectiveness of synthetic analogs of estrone structurally similar to EM but containing no alkylating moiety. The presence of a carbamate group conjugated to a homocyclic or heterocyclic ring at the 2 or 3 positions is the common structural property of EM , nocodazole, and other microtubule inhibitors (Gupta 1986). Other steroid alkylating agents also have a carbamate-ester group at the 3 position of the steroid A ring. These antileukemic agents may have been microtubule inhibitors as well (Catsoulacos *et al.*, 1979). Interestingly, when EM was in development other compounds with similar structures were most effective when a carbamate group was conjugated at position 2 or 3 of the heterocyclic or homocyclic ring (Leclercq *et al.*, 1978). These agents, unfortunately, were never tested for antimicrotubule action, nor were these evaluated for antiproliferative action against malignant glioma.

The estrone analogs we had synthesized were all inhibitors of DNA synthesis in glioma cell cultures . The carbamate at position 2 of the steroid A-ring (JE208) was the most active compound. A carbamate at the four position (JE205) slightly reduces this antiproliferative effect. Cyclization of the ethyl carbamate of JE208 to 2,3-oxozonaly estrone (JE212) reduces this action just slightly. Finally, the replacement of the ester group of JE208 with an acetate (JE213) also decreases the antiproliferative action. Although we have not demonstrated that the estrone analogs and EM exert their antiproliferative effect via the same mechanism, we know of no other reason that these analogs should inhibit DNA synthesis. In addition the morphological alterations of glioblastoma cells caused by JE208 were similar to those caused by EM (Figure 29). Although the effect is less dramatic than when cells are treated with EM, exposure to JE208 results in a rounding-up of cells and a decrease in cytoplasmic projections.

The therapeutic potential of microtubule inhibitors in the management of glioblastoma is indicated by the elevated levels of microtubules found in these neoplasms (Lantos, 1977). Taxol, a new antimicrotubule agent has been demonstrated to be highly effective in the

management of ovarian cancer (McGuire *et al.*, 1989). Recently, taxol was used *in vitro* with EM against prostatic carcinoma cell lines (Speicher, Barone, and Tew, 1992). EM and taxol produced a greater than additive effect on the inhibition of cell survival in prostatic cell lines. Interestingly, vinblastine did not enhance the cytotoxic effect of taxol. It has also been determined that EM can inhibit invasion of DU-145 prostate carcinoma cells *in vitro* (Mareel *et al.*, 1988). This would be an important quality of an antiglioma agent since invasion of normal brain parenchyma is a devastating characteristic of astrocytomas. Since prostatic carcinoma cells display the metaphase arrest seen in glioma cells (Sheridan, Speicher, and Tew, 1991), there is no reason to believe that EM could not inhibit the *in vitro* invasion of glioma.

Tamoxifen and estramustine are a potent combination

We have confirmed reports that the triphenylethylene derivative tamoxifen is a potent inhibitor of glioma DNA synthesis. The concentrations we used in culture approximate that attainable in breast cancer patients. Most of the early studies on tamoxifen plasma levels measured concentrations of around 2 μg/ml after chronic tamoxifen administration (Daniel *et al.*, 1979; Murphy *et al.*, 1987). A recent study reported preliminary results on the use of oral tamoxifen in patients with glioma refractory to conventional therapy. Of 32 patients with advanced glioblastoma, 7 remained neurologically stable without radiographic evidence of tumor progression for 6 or more months (Vertosick *et al.*, 1992). Unfortunately these results are difficult to interpret due to the lack of a control group and the arbitrary definition of a positive response to tamoxifen (stable disease for 6 months).

Our data also supported the hypothesis that EM action is independent of estrogen receptors (ER). If EM functions via the ER, tamoxifen would decrease the inhibition of DNA synthesis caused by EM. Although it is widely believed that EM is a microtubule inhibiting agent that functions independent of ER, a recent report found that the presence of ER correlates with the antiproliferative action of EM (Pavelic, Zgradic, and Pavelic, 1991).

This study examined human mammary, prostatic, renal, and uterine carcinoma cultures. A possible explanation for their observation is that ER may correlate with the presence of EMBP. Also, ER may be present in more differentiated tumors more susceptible to chemotherapy. One other report found that increasing levels of estradiol abolished the antiproliferative effect of estramustine in gastrointestinal cancer cell lines (Harrison *et al.*, 1990). This may not be a result of ER blockade by estrogen, but rather estrogenic stimulation of the cancer cell lines.

PKC is an attractive target for the therapy of malignant glioma as well as some other tumors. Malignant glial cell lines contain large amounts of PKC (Couldwell *et al.*, 1991) and most gliomas express immunoreactive PKC as well (Reifenberger, Deckert, and Weschler, 1989). In addition, PKC is expressed in developing glia and malignant glia but is poorly expressed in adult quiescent glial cells (Bhat, 1989). Over-expression of PKC in transfected fibroblasts leads to enhanced tumorigenicity (Persons *et al.*, 1988) and increased growth rate (Housey *et al.*, 1988). PKC inhibition blocks v-*src* and v-*fps* induced expression of a transformation-related *9E3* gene (Spangler *et al.*, 1989).

We found the combination of TAM and EM to be more effective in inhibiting DNA synthesis in glioblastoma cell cultures than either agent individually. It appears as if this effect is additive rather than synergistic. Although this would indicate the two agents exert their effects through independent targets, there still may be a synergistic component to their combined action. Tamoxifen may be well suited for combination therapy with EM because PKC is implicated in the control of microtubule function. A binding site for tamoxifen has been located on protein kinase C (O'Brian, Housey *et al.*, 1988). Activation of PKC induces the formation of microtubules in cytoplasmic processes of human fibroblasts (Tint *et al.*, 1991) and inhibition of PKC by sphingosine arrests neurite outgrowth (Hall *et al.*, 1988). Also, PKC has been demonstrated to catalyze the phosphorylation of microtubule associated proteins (MAPs) (Hoshi *et al.*, 1987, 1988). This can lead to increased DNA synthesis (Shaw, Chou, and Anand, 1988). As noted previously, malignant glial cells contain

abundant microtubules and MAPs (Lantos, 1977; Koszka, Leichfried, and Wiche, 1985). We found distinct morphological alterations of glioblastoma cells treated with TAM which may indicate action on the cytoskeleton (Figure 18c). In order to better clarify the mechansims of the combined action of EM and TAM we will use a wider concentration range for the two agents to generate an isobologram for analysis.

Curiously, 5.0 µg/ml TAM seemed to inhibit the proliferation of a larger percentage of J889H cells in Figure 16 than seen in Figure 13. We are unable to explain this effect except that a different stock of TAM was used which may have been more potent. However, it is possible that the cells were proliferating at a greater rate in the second experiment (Figure 16) which would render them more susceptible to the inhibitory effects of TAM. In support of this, the control CPM is slightly greater in the second experiment but this may simply be due to a technical artifact.

Some clinical side-effects of EM are estrogen-related, presumably due to increased levels of circulating estrogens and estrogen metabolites (Andersson *et al.*, 1981). Fossa and co-workers (1977) demonstrated an increase in the circulating levels of estrogen in men given estramustine phosphate for the treatment of prostate cancer. Fredholm *et al.* (1974) noticed that EM , when administered to rodents, had a uterotropic effect although it was two orders of magnitude weaker than estradiol. Daehlin and colleagues (1986) also detected an increase in plasma estradiol levels in prostatic carcinoma patients after six months administration of 9.2 mg/kg estramustine phosphate. Circulating estradiol increased from 0.08 nM before treatment to 27.9 nM after treatment. Gunnarsson and co workers (1981) demonstrated a dose dependent increase in circulating estradiol levels in patients treated with increasing oral doses of estramustine phosphate. As the dose of EM increased from 70 to 560 mg/day, the estradiol levels in the plasma increased from a mean of 0.7 ng/ml to a mean of 7.1 ng/ml.

We investigated the effect of combining EM and TAM on the proliferation of cultured human glioblastoma cells since these agents may possess synergistic actions against microtubules. This combination also targets distinct physiologic mechanisms which may

enhance the antiproliferative action of either agent when used alone. It appears that these additive effects via separate cellular targets predominate the combined action of the two drugs. In addition, TAM may benefit patients suffering from estrogenic side-effects of EM. Wtih a disease as devestating as glioblastoma, new combinations of drugs with few and different dose limiting toxicities are desperately in need. Further research studying the development of synergistic drugs acting on microtubule function via distinct mechanisms may prove to be of value in other malignancies as well.

Prior exposure of glioblastoma cells to estramustine enhances the cytotoxicity of bleomycin

Attempts to enhance the effects of combination chemotherapy by sequential administration of agents have been reported previously (Nicolini, 1976; Shimuzu *et al.*, 1980). One group achieved promising results when they pretreated patients with various tumors with vincristine followed by procarbazine, cyclophosphamide, or ara-C (Pouillart *et al.*, 1975). Other groups used hydroxyurea, ara-C, and VM26 as synchronizing agents (for review see Van Putten, Keizer, and Mulder, 1976). The rationale in these studies was to synchronize tumor cells with one agent and then release the cells into a phase of the cell cycle in which the next agent is most cytotoxic. In order to synchronize cells *in vivo* patients must be treated with the first agent for long enough to synchronize a significant proportion of the tumor population. In cancers with slow doubling times, this may be impossible due to the toxic effects of the synchronizing agent. Since estramustine has been associated with very little morbidity in patients treated for prostate cancer, we thought it was well suited as a synchronizing agent since it could be given to patients for long periods. In addition, it may also accumulate specifically in tumor cells due to the presence of EMBP.

Barranco and colleagues (1982) demonstrated the enhanced cytotoxicity of bleomycin when CHO cells were synchronized in G2/M phase (Figure 30). When CHO cells were synchronized with dianhydrogalactitiol (DAG) and released, cells would progress through G2/M phase 18 hours later. If these cells were exposed to bleomycin at this time, DNA

synthesis was more inhibited than at any other time. If cells were synchronized in all phases of the cell cycle (Barranco and Humphrey, 1971), those in G2/M were most susceptible to bleomycin (Figure 31). When we pretreated glioblastoma cells with estramustine, we noted a significant increase in cells in G2/M phase. Following this, the application of bleomycin to the cell cultures resulted in enhanced cytotoxicity of bleomycin.

It appears as if the increased antiproliferative effect as a result of the combination of EM and BLM is an additive effect. In the concentrations used in this study the agents are likely acting on separate cellular mechanisms. However, it is still possible that the enhanced cytotoxicity resulting from the combination of agents may, especially at concentrations not used in this report, be due to EM induced increase of cells in G2/M, although we cannot, at this time, prove this. EM can also inhibit glutathione-S-transferase (GST) activity in DU-145 cells (Tew, Woodworth, and Stearns, 1986; Tew and Stearns, 1987). Bleomycin has been shown to have enhanced cytotoxicity in CHO variants that possess little or no GST activity (Giaccia *et al.*, 1991). Therefore the increase in DNA synthesis inhibition by the combination of EM and BLM may be due to non-cell cycle specific effects. Regardless of mechanism, the combination of BLM has been shown to be a highly effective in vito inhibitor of malignant glioma proliferation. Further research will assess the important clinical utility of this data.

Urgent need for effective therapy against malignant glioma

We are currently testing other agents in combination with estramustine since we believe the potential for successful chemotherapy in patients with glioblastoma lies with combination chemotherapy. We are also studying the combination of estrone analogs and various conventional agents a similar fashion. Resistance to EM can develop in DU145 prostate cancer cells (Speicher *et al.*, 1991) which emphasizes the importance of combination therapy. *In vitro* drug sensitivity testing for malignant glioma has become an acceptable method for the evaluation of new agents as well as a method for determining a patient's

tumor sensitivity to a specific drug (Nikkhah *et al.*, 1992). Our cell culture system provides a reliable method of preclinical evaluation of chemotherapeutic agents against glioblastoma.

The need for more effective therapy in the management of malignant glioma is evident in a recent large multicenter trial of a promising antiglioma drug (Schold *et al.*, 1993). Diaziquone (AZQ) was evaluated against the current standard BCNU in 251 patients randomized after radiation therapy (78% had some prior surgical resection). The median survival after randomization for patients with glioblastoma over the age of 45 was only 37 weeks without a significant difference between the two agents. Patients with GBM or age less than 45, or age less than 45 and with anaplastic astrocytoma, fared better with median survivals of 61 and 147 weeks, respectively. There was no difference between those treated with AZQ and those treated with BCNU. Again, cancer research has produced an agent that is no more effective than a drug used for almost 30 years in the treatment of glioblastoma. Clearly, more effective agents are needed.

The future of therapy for glioblastoma will likely come from immunology (see Ingram *et al.*, 1990 and Nitta *et al.*, 1990). Until then, however, the elucidation of various molecular targets for chemotherapeutic attack will remain an important task for glioblastoma researchers. New agents aimed at specific proteins and functions of malignant gliomas in combination with surgery and radiation are currently the only hope for patients with the dismal diagnosis of glioblastoma.

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TABLE 1. *Sex of patient and histolgy of operative tumor specimens.*

Tumor	Sex	Histology
J889H	M	GBM
H1289G	F	GBM
JMz	M	Anaplastic Oligodendroglioma
AM	M	GBM
SZ	M	GBM
WG	M	GBM
WP	M	BRAINSTEM GBM
SC	M	EPENDYMOMA
RB	M	GBM
RC	F	GBM
JML	M	GBM
AMe	M	GBM
LS	F	cellular astrocytoma

GBM, glioblastoma; M, male; F, female

TABLE 2. *The presence of hormone receptor protein in tumor tissue from operative specimens.*

TUMOR	AR fmol/mg protein	ER fmol/mg protein	PR fmol/mg protein
H1289G	65.9	0	74.0
WG	35.7	0	9.7
IH	0	26.9	11.9
AM	26.4	21.0	20.4
JMz	0	29.0	2.3

androgen receptor, AR; estrogen receptor, ER; progesterone receptor, PR

TABLE 3. *Percent inhibition of DNA synthesis by estramustine (EM) and tamoxifen (TAM) and their combination.*

TUMOR	TAM 5.0 ug/ml	EM 2.5 mg/ml	TAM+EM
HS683	49	65	92 *
J889H	48	71	87 *
H1289G	95	81	98 *
SZ	81	69	95 **

* significantly greater than TAM and EM alone ($p<0.0001$)
** significantly greater than TAM and EM alone ($p<0.001$)

TABLE 4: *Percentage of cells in G2/M phase after exposure to estramustine for 24 hours*

Cells	Treatment	% G2/M (mean) *
HS683	Control EM 10^{-5}M	28 50
H1289G	Control EM 10^{-5}M	23 53
J889H	Control EM 10^{-5}M	30 41

* significant at $p<0.019$ (paired t-test) when compared to control values in all cell cultures
n=3 for all groups

FIGURE LEGENDS

Figure 1. The structures of estrone, estromustine (the active metabolite of estramustine), and various synthetic analogs of estrone synthesized in the laboratory of Dr. Jan Zielinski. Note the absence of an alkylating moiety on the estrone analogs.

Figure 2 AM glioblastoma cells, in media containing 10% gelded-horse, serum were exposed to 10^{-7}M concentrations of ß-estradiol, progesterone, or DHT or 0.1% ethanol control for 72h before 4h incubation with 3H-thymidine. Error bars depict SEM. For each treatment group, n=4. No significant effect was observed (Student's T-Test, $p > 0.05$).

Figure 3 H1289G glioblastoma cells, in media containing 10% gelded horse serum, were exposed to 10^{-6}M concentration of ß-estradiol, progesterone, or DHT or 0.1% ethanol control for 72h before 4h incubation with 3H-thymidine. Error bars depict SEM. For each treatment group, n=4 No significant effect was observed (Student's T-Test, $p > 0.05$).

Figure 4. J889H and RB glioblastoma cells were exposed to EM in increasing concentrations for 24h before 3H-thymidine incorporation analysis. The effect of EM is dose related in both cell cultures (ANOVA, $p < 0.05$). For each concentration n=6. Error bars depict SEM.

Figure 5. JMl and AM glioblastoma cells were exposed to EM in increasing concentrations for 24h before 3H-thymidine incorporation analysis. The effect of EM is dose related in both cell cultures (ANOVA, $p < 0.05$). For each concentration n=6. Error bars depict SEM.

Figure 6. J889H glioblastoma cells were exposed to increasing concentrations of EM for 24h before 3H-thymidine incorporation studies. Estramustine inhibits DNA synthesis in

J889H in a dose dependent fashion ($p < 0.001$), ANOVA). For each concentration n=6. Error bars depict SEM.

Figure 7. Exposure of J889H glioblastoma cells to EM and synthetic estrone analogs at a concentration of 10^{-5}M for 24h. All agents significantly inhibited DNA synthesis with EM being most effective followed by JE208, JE212, JE 205, and JE 213 ($p < 0.01$, Student's T-Test). For all groups n=6.

Figure 8. Exposure of J889H glioblastoma cells to EM and synthetic estrone analogs at a concentration of 10^{-5}M for 4h. All agents significantly inhibited DNA synthesis with EM being most effective followed by JE208, JE212, JE 205, and JE 213 ($p < 0.05$, Student's T-Test). For all groups n=6.

Figure 9. Tamoxifen inhibits DNA synthesis in cultured glioblastoma. Cells were exposed to tamoxifen at a concentration of 0.2 or 5.0 µg/ml for 48h. All cultures displayed reduced thymidine incorporation at the higher concentration ($p < 0.001$), but the lower concentration of tamoxifen had no effect on proliferation ($p > 0.05$, Student's T-Test). For each concentration n=6 except control (n=12). Error bars depict SEM.

Figure 10, Exposure of J889H glioblastoma cells to increasing concentrations of tamoxifen for 24h. Note the large decrease in DNA synthesis when the concentration of tamoxifen is doubled from 2.5 µg/ml to 5.0 µg/ml. The inhibitory effect is dose related ($p < 0.05$, ANOVA) but only 2.5 and 5.0 µg/ml significantly inhibit DNA synthesis $p<0.05$, Student's T). For each concentration n=6 except control (n=12). Error bars depict SEM.

Figure 11. J889H glioblastoma cells were exposed to increasing concentrations of tamoxifen for 24h. 3[H]-thymidine incorporation is expressed as percent of control. A dose

related inhibition of DNA synthesis is noted for 12 and 24 hours ($p<0.05$, ANOVA) but not 0 and 4 hours. Five µg/ml tamoxifen significantly inhibited DNA synthesis for all durations except the immediate exposure ($p <0.05$, Student's T). For each concentration n=6 except control (n=11). Error bars depict SEM.

Figure 12. Combining estramustine (EM) 2.5 µg/ml ($5.68x10^{-6}M$) and tamoxifen 5.0 µg/ml (TAM) in culture for 24h significantly inhibit DNA synthesis in HS683 glioblastoma cell line. The combination is more efficacious than either agent alone ($p <0.001$, Student's T). For each group n=6. Error bars depict SEM.

Figure 13. Combining estramustine (EM) 2.5 µg/ml ($5.68x10^{-6}M$) and tamoxifen 5.0 µg/ml (TAM) in culture for 24h significantly inhibit DNA synthesis in J889H glioblastoma cells. The combination is more efficacious than either agent alone ($p <0.001$, Student's T). For each group n=6. Error bars depict SEM.

Figure 14. Combining estramustine (EM) 2.5 µg/ml ($5.68x10^{-6}M$) and tamoxifen 5.0 µg/ml (TAM) in culture for 24h significantly inhibit DNA synthesis in SZ glioblastoma cells. The combination is more efficacious than either agent alone ($p <0.001$, Student's T). Error bars depict SEM.

Figure 15. Combining estramustine (EM) 2.5 µg/ml ($5.68x10^{-6}M$) and tamoxifen 5.0 µg/ml (TAM) in culture for 24h significantly inhibit DNA synthesis in H1289G glioblastoma cells. The combination is more efficacious than either agent alone ($p <0.001$, Student's T). For each group n=6. Error bars depict SEM.

Figure 16. Decreasing the concentration of EM to 1.0 µg/ml ($2.27x10^{-6}M$), with tamoxifen still 5.0 µg/ml, still results in an enhancement of tamoxifen inhibition of DNA synthesis in

J889H glioblastoma cells. The combination is more effective than either agent alone ($p < 0.05$, Student's T). For each group n=6. Error bars depict SEM.

Figure 17. When the concentration of tamoxifen is decreased from 5.0 to 2.5 μg/ml the combination of EM and tamoxifen is no longer more effective in inhibiting DNA synthesis than EM alone. For each group n=6. Error bars depict SEM.

Figure 18a. H1289G glioblastoma cells after 24 hours exposure to control media (0.1% EtOH and 0.1% DMSO). Note the cytoplasmic processes typical of malignant glial cells well as the different oblong shapes.

Figure 18b. H1289G glioblastoma cells after 24 hours exposure to media containing tamoxifen (TAM) 5.0 μg/ml in EtOH (0.1%) and 0.1% DMSO. The cells appear more tubular than control and almost bipolar.

Figure 18c. H1289G glioblastoma cells after 24 ours exposure to media containing estramustine (EM) 2.5 μg/ml (5.68×10^{-6}M) in DMSO (0.1%) and 0.1% EtOH. The cells have a more rounded appearance typical of cells arrested in G2/M. Cytoplasmic processes are retracted forming "blebs" at the cell surface.

Figure 18 d. H1289G glioblastoma cells after 24 hours exposure to media containing tamoxifen (TAM) in EtOH (0.1%) and estramustine (EM) 2.5 μg/ml (5.68×10^{-6}M) in 0.1% DMSO. The cells are few in number and appear almost nonviable. Their morphology is more of a rounded-up appearance but with few surface blebs.

Figure 19a. DNA histogram for HS683 cells exposed to 10-5M EM for 24 hours. Note the increase in the G2/M fraction in cells exposed to EM.

Figure 19b. DNA histogram for J889H cells exposed to 10-5M EM for 24 hours. Note the increase in the G2/M fraction in cells exposed to EM.

Figure 20. J889H cells were exposed to 10-6M EM for 24 hours. The top histogram represents control and the bottom histogram represents EM treated cells. The was no difference in the two groups.

Figure 21. H1289G cells were exposed to EM 10-5M (top histogram) or control (bottom) for 4 or 24 hours. There was no difference in the control groups. Cells exposed to 4 hours of EM (top, darker line) did not differ from control. Only cells exposed for 24 hours to EM (top, lighter line) showed the characteristic increase in G2/M fraction.

Figure 22. Dose response curve of increasing concentrations of bleomycin (BLM). J889H glioblastoma cells were exposed to BLM for 4 or 24 hours before thymidine incorporation analysis. Since 24 hours appeared to be excessive, we decided to incubate for only four hours in the combination studies. For all concentrations n=6.

Figure 23. HS683 glioblastoma cell line was pretreated for 24 hours with 10^{-5}M or $5x10^{-6}$M estramustine (EM) or 0.1% DMSO control. The media was withdrawn and the cells were then exposed to bleomycin (BLM) at a concentration of 1μg/ml for 4 hours. Thymidine incorporation was then assessed. Both EM and BLM inhibit DNA synthesis in a dose dependent fashion. When cells are pretreated with EM, BLM more potently inhibits DNA synthesis when compared to pretreatment with control (Student's T-test, $p < 0.01$). For all groups n=6. Error bars depict SEM.

Figure 24. H1289G glioblastoma cells were pretreated for 24 hours with 10^{-5}M or $5x10^{-6}$M estramustine (EM) or 0.1% DMSO control. The media was withdrawn and the cells were then exposed to bleomycin (BLM) at a concentration of 1μg/ml for 4 hours. Thymidine incorporation was then assessed. Both EM and BLM inhibit DNA synthesis in a dose dependent fashion. When cells are pretreated with EM, BLM more potently inhibits DNA synthesis when compared to pretreatment with control (Student's T-test, $p < 0.01$). For all groups n=6. Error bars depict SEM.

Figure 25. J889H glioblastoma cells were pretreated for 24 hours with 10^{-5}M or $5x10^{-6}$M estramustine (EM) or 0.1% DMSO control. The media was withdrawn and the cells were then exposed to bleomycin (BLM) at a concentration of 1μg/ml for 4 hours. Thymidine incorporation was then assessed. Both EM and BLM inhibit DNA synthesis in a dose dependent fashion. When cells are pretreated with EM, BLM more potently inhibits DNA synthesis when compared to pretreatment with control (Student's T-test, $p < 0.01$). For all groups n=6. Error bars depict SEM.

Figure 26. Correlation between HS683 cells arrested in G2/M by 10^{-5}M estramustine (EM) and DNA synthesis inhibition by bleomycin BLM after pretreatment with 10^{-5}M EM. The lighter shaded bars represent percentage of all cells in G2/M phase (measured by flow cytometry) after exposure to EM or control. The darker bars represent percent decrease in 3[H]-thymidine incorporation. As cells are arrested in G2/M by EM, BLM becomes a more potent inhibitor of DNA synthesis.

Figure 27. Correlation between H1289G cells arrested in G2/M by 10^{-5}M estramustine (EM) and DNA synthesis inhibition by bleomycin BLM after pretreatment with 10^{-5}M EM. The lighter shaded bars represent percentage of all cells in G2/M phase (measured by flow cytometry) after exposure to EM or control. The darker bars represent percent decrease in

3[H]-thymidine incorporation. As cells are arrested in G2/M by EM, BLM becomes a more potent inhibitor of DNA synthesis.

Figure 28. Correlation between J889H cells arrested in G2/M by 10^{-5}M estramustine (EM) and DNA synthesis inhibition by bleomycin BLM after pretreatment with 10^{-5}M EM. The lighter shaded bars represent percentage of all cells in G2/M phase (measured by flow cytometry) after exposure to EM or control. The darker bars represent percent decrease in 3[H]-thymidine incorporation. As cells are arrested in G2/M by EM, BLM becomes a more potent inhibitor of DNA synthesis.

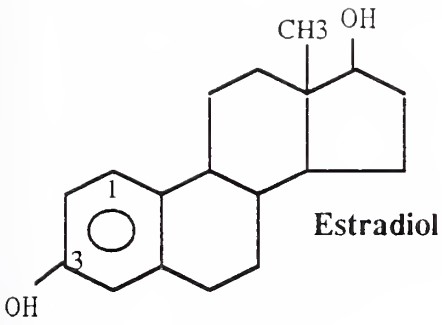
Figure 29a. J889H cells exposed to control media for 24 hours. Note the characteristic cytoplasmic processes.

Figure 29b. J889H cells exposed to EM at a concentration of 10-5M for 24 hours. Note the typical rounding-up of cells and cell surface blebs.

Figure 29c. J889H cells exposed to JE208 at a concentration of 10-5M for 24 hours. There are more rounded cells than in those treated with control media, a picture typical for EM treated cells. Also, note the absence of long cytoplasmic processes.

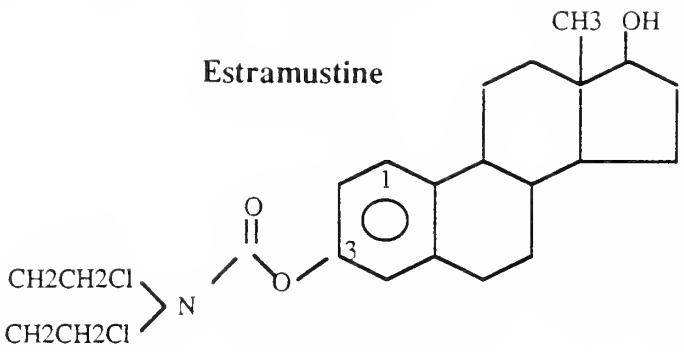
Figure 30. This graph adapted from Barranco *et al.* (1981), depicts the effect of synchronizing Chinese hamster ovary (CHO) cells with dianhydrogalactitol and then releasing them. 18 hours later the majority of cells are in G2/M phase. When bleomycin is added to cultures at timed intervals, it is most cytotoxic at 18 hours.

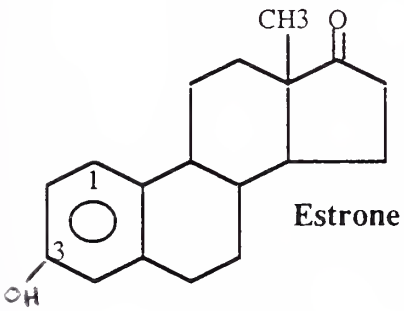
Figure 31. CHO cells were synchronized in various cell cycle phases with double-thymidine block. Cells in G2/M phase were most susceptible to bleomycin when they were in G2/M.



Estradiol

Estramustine





Estrone

Estromustine

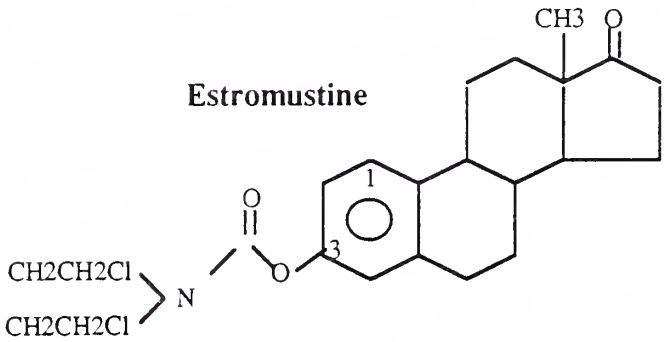
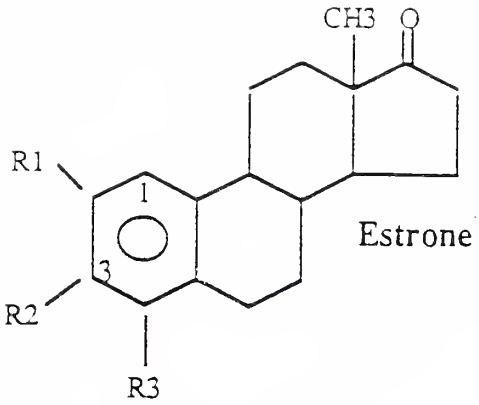
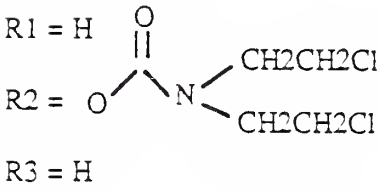


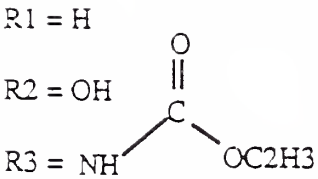
Figure 1a.



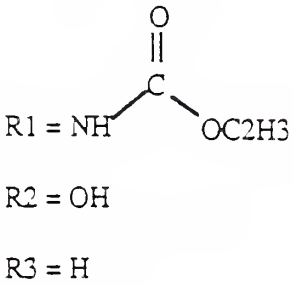
Estrone



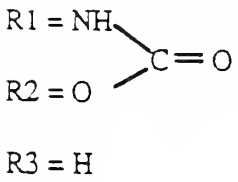
Estromustine



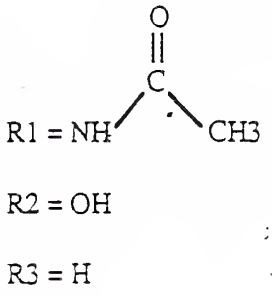
JE205



JE208



JE 212



JE213

Figure 1b.

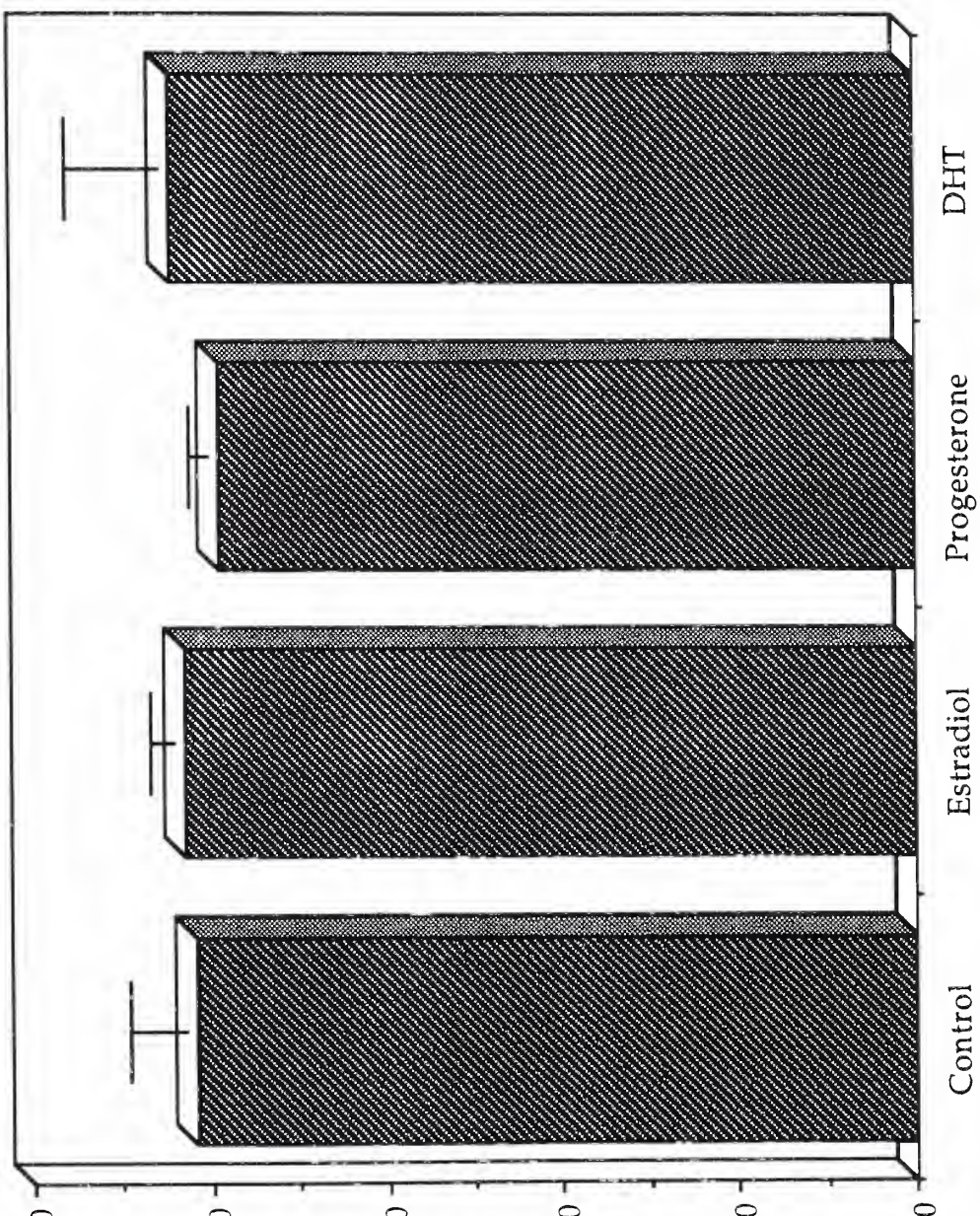

Lack of effect on 3[H]-thymidine incorporation of H1289G glioblastoma cells after 72h exposure to various sex hormones (concentration 10-7M)
CPM
150000
120000
90000
60000
30000
0
Control
Estradiol
Progesterone
DHT

Figure 2.

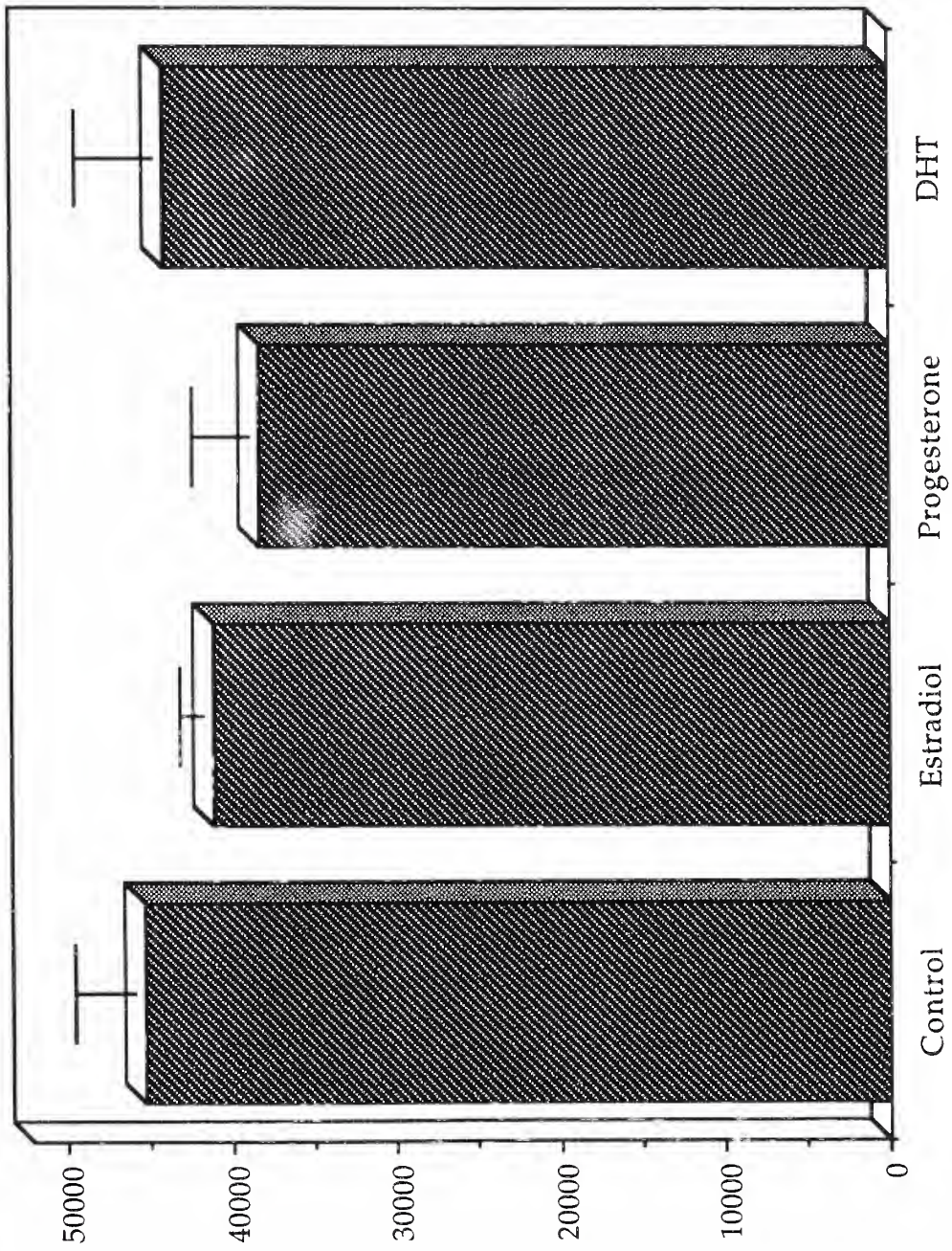

Lack of effect on 3[H]-thymidine incorporation of AM glioblastoma cells
after 72h exposure to various sex hormones (concentration 10-6M)
CPM
50000
40000
30000
20000
10000
0
Control
Estradiol
Progesterone
DHT

Figure 3.

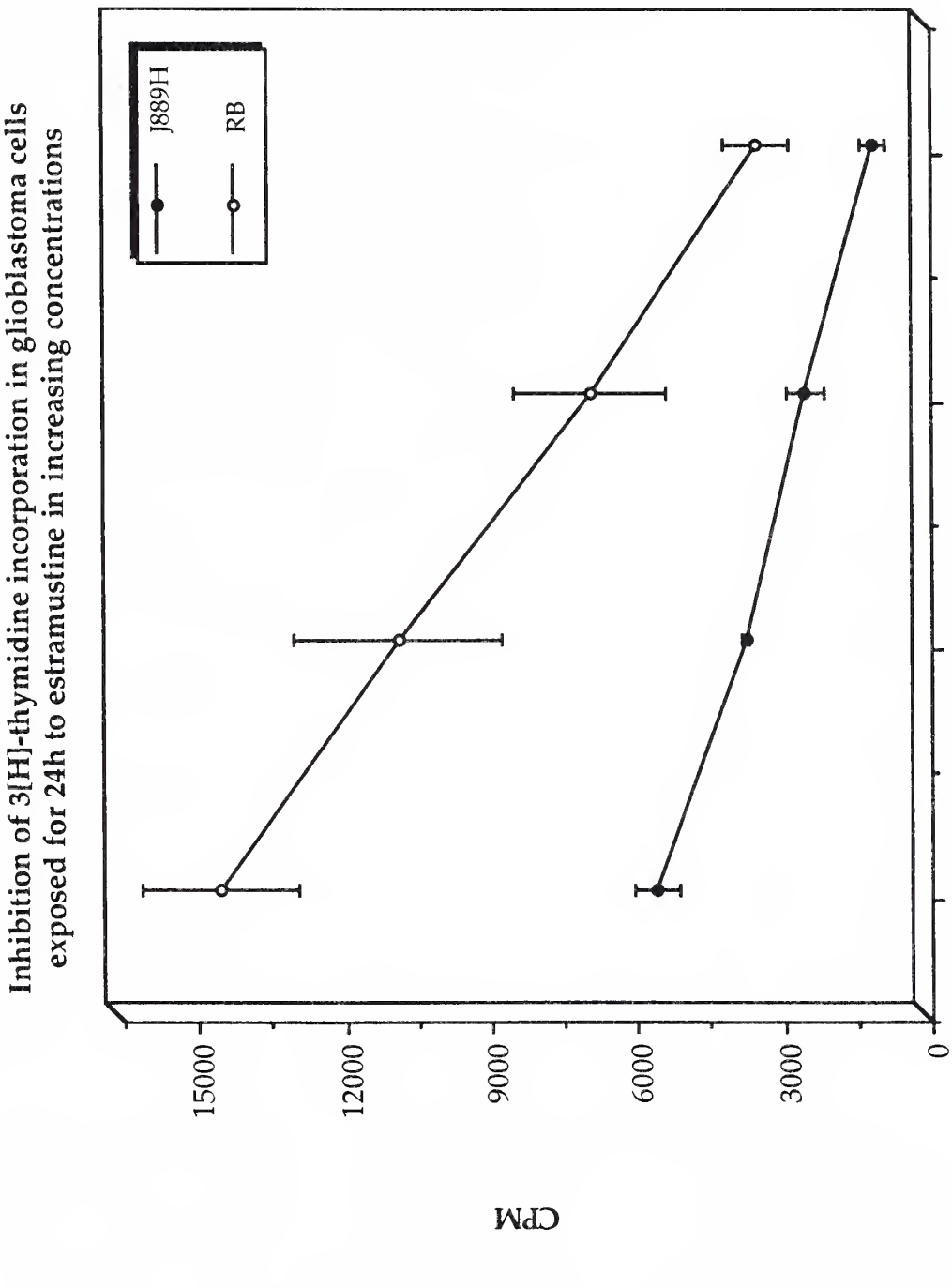

Inhibition of 3[H]-thymidine incorporation in glioblastoma cells
exposed for 24h to estramustine in increasing concentrations
J889H
RB
CPM
15000
12000
9000
6000
3000
0
0
1.0
2.5
5.0
Concentration of estrammustine (ug/ml)

Figure 4.

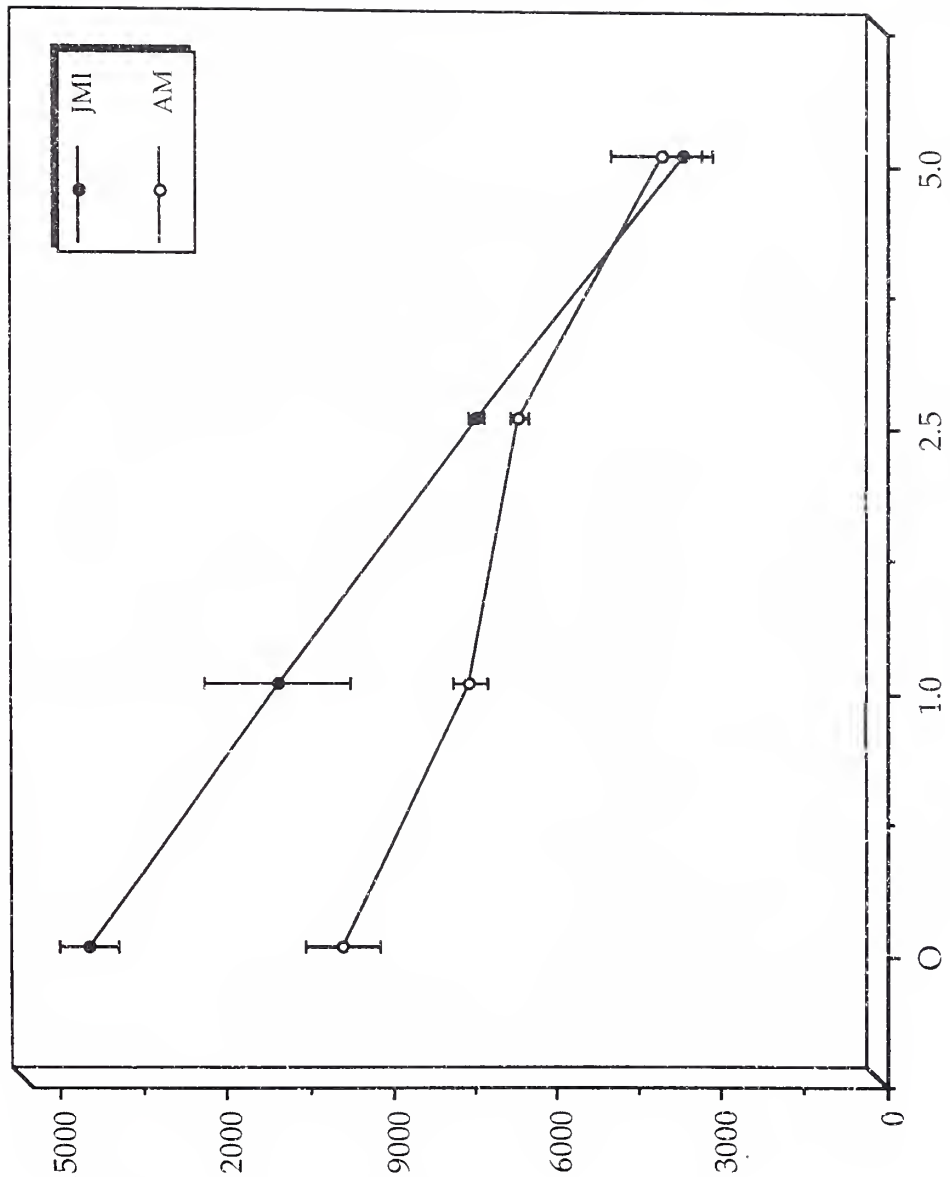

Inhibition of 3[H]-thymidine incorporation in glioblastoma cells
exposed for 24h to increasing concentrations of estramustine
JMI
AM
CPM
15000
12000
9000
6000
3000
0
0
1.0
2.5
5.0
Concentration of estrammustine (ug/ml)

Figure 5.

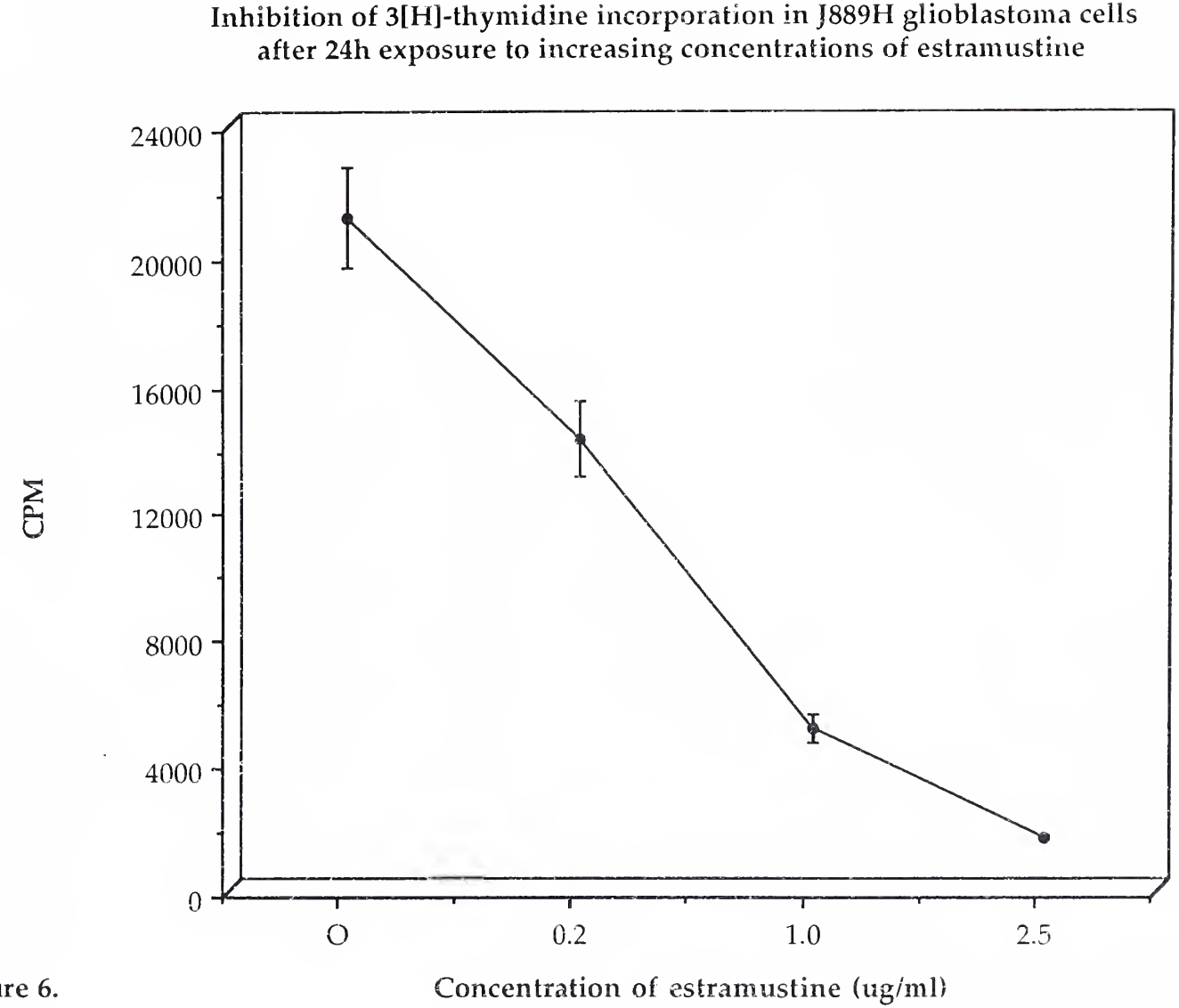

Inhibition of 3[H]-thymidine incorporation in J889H glioblastoma cells
after 24h exposure to increasing concentrations of estramustine
CPM
24000
20000
16000
12000
8000
4000
0
O
0.2
1.0
2.5
Concentration of estramustine (ug/ml)

Figure 6.

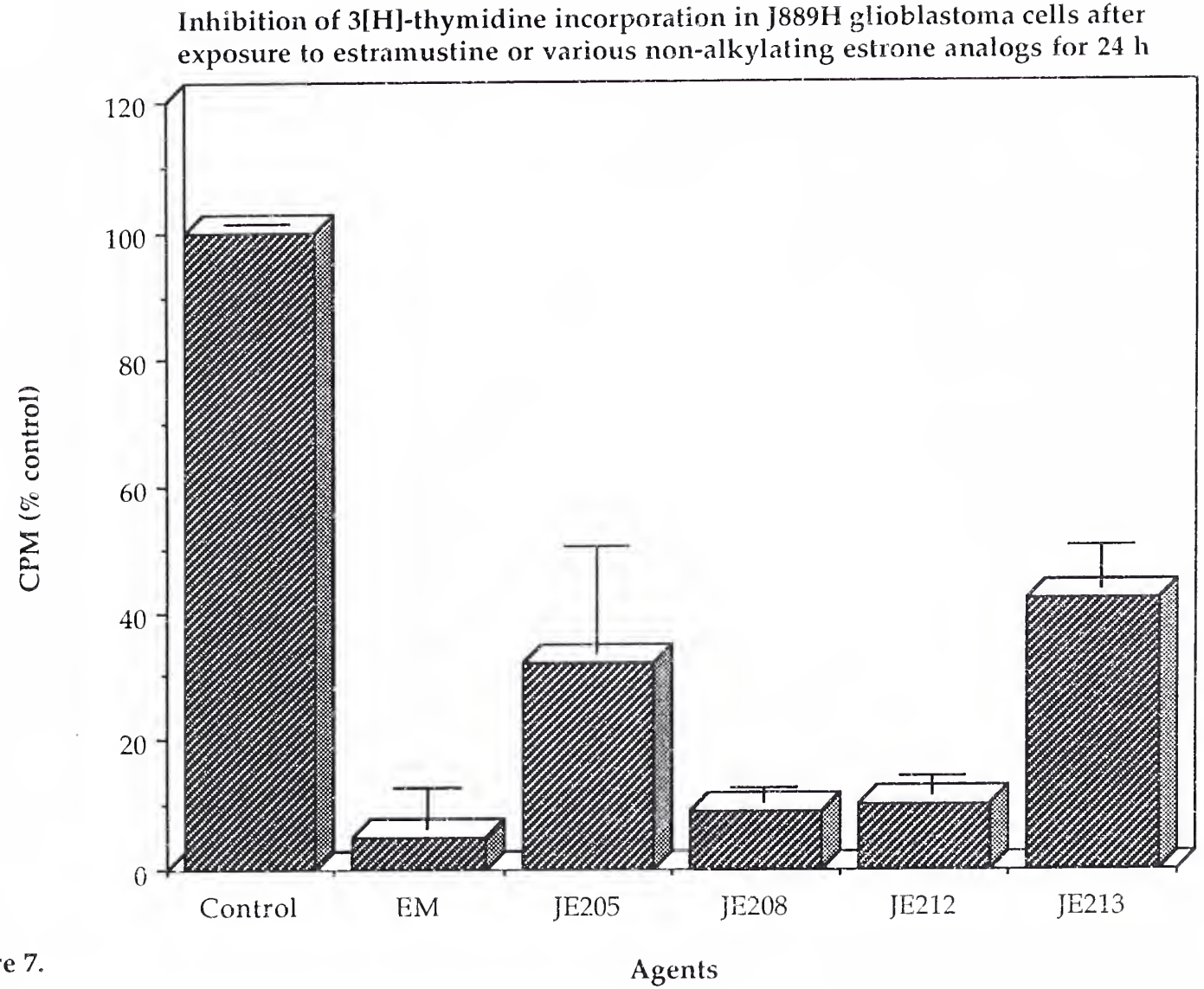

Inhibition of 3[H]-thymidine incorporation in J889H glioblastoma cells after exposure to estramustine or various non-alkylating estrone analogs for 24 h
CPM (% control)
120
100
80
60
40
20
0
Control
EM
JE205
JE208
JE212
JE213
Agents

Figure 7.

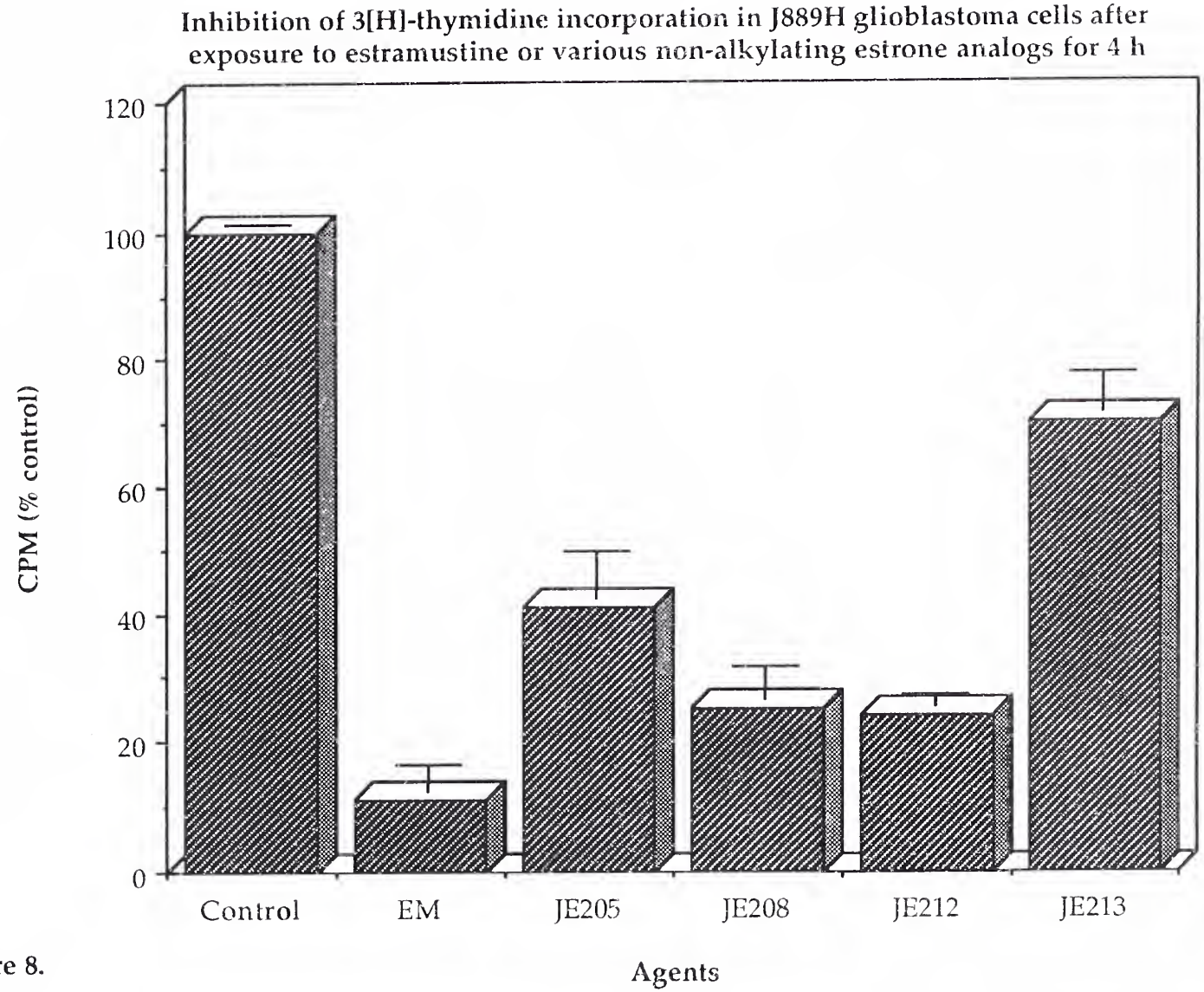

Inhibition of 3[H]-thymidine incorporation in J889H glioblastoma cells after exposure to estramustine or various non-alkylating estrone analogs for 4 h
CPM (% control)
120
100
80
60
40
20
0
Control
EM
JE205
JE208
JE212
JE213
Agents

Figure 8.

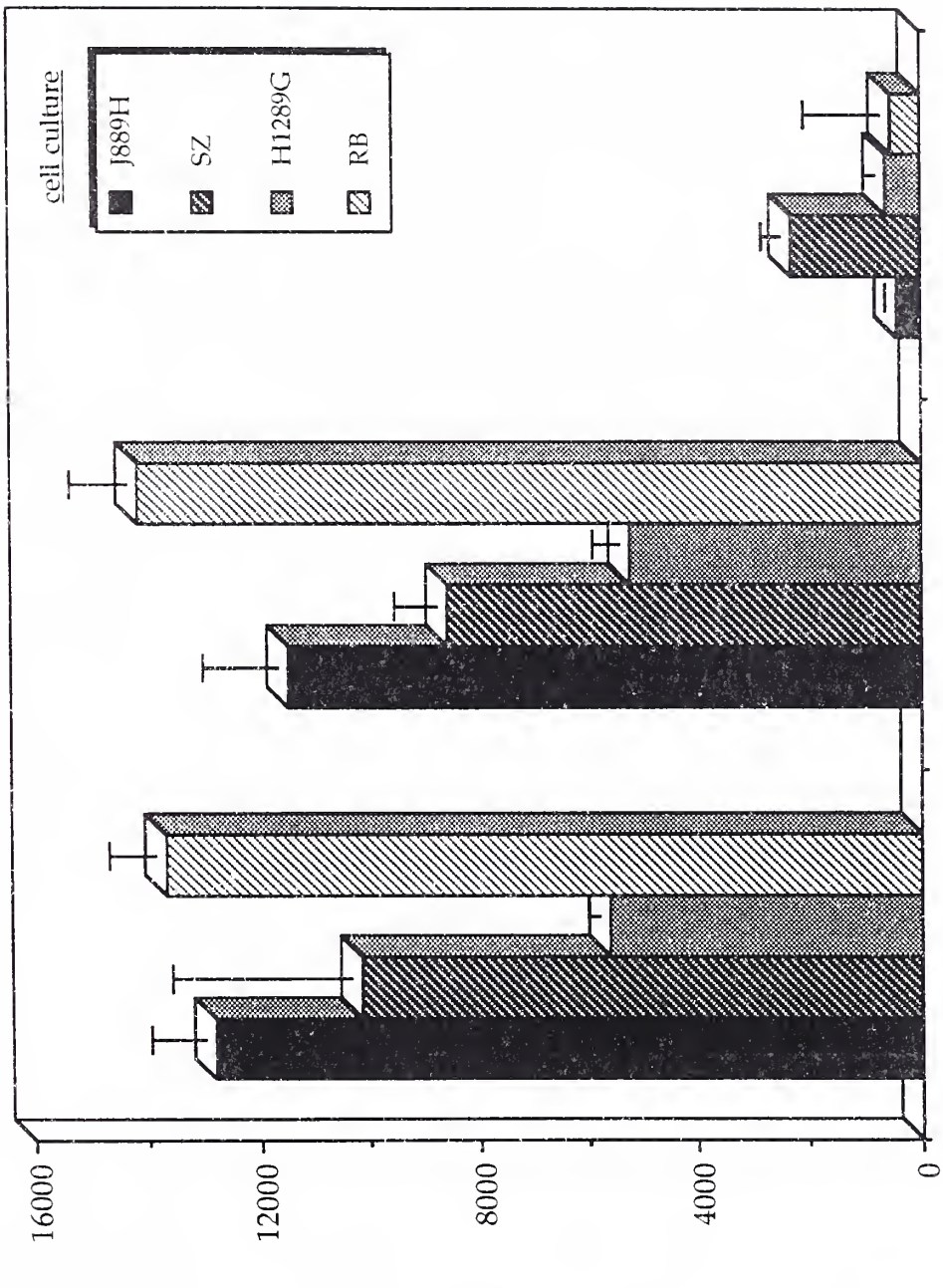

Exposure of glioblastoma cell cultures to tamoxifen for 24 hours
cell culture
J889H
SZ
H1289G
RB
CPM
16000
12000
8000
4000
0
0
0.2
5.0
Tamoxifen (ug/ml)

Figure 9.

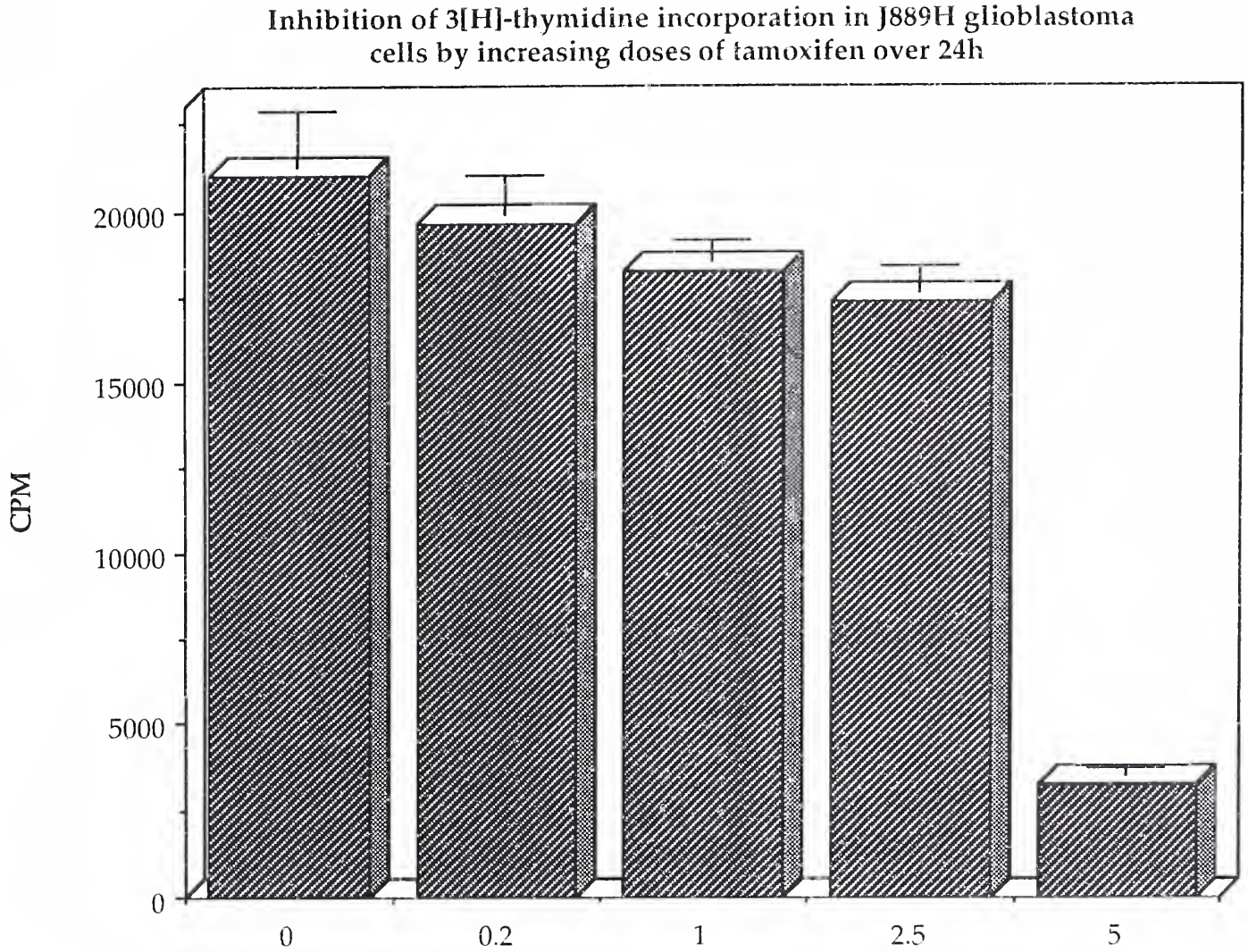

Inhibition of 3[H]-thymidine incorporation in J889H glioblastoma cells by increasing doses of tamoxifen over 24h
CPM
20000
15000
10000
5000
0
0
0.2
1
2.5
5
Tamoxifen (ug/ml)

Figure 10.

Effect of dose and time on the inhibition of 3[H]-thymidine incorporation by tamoxifen

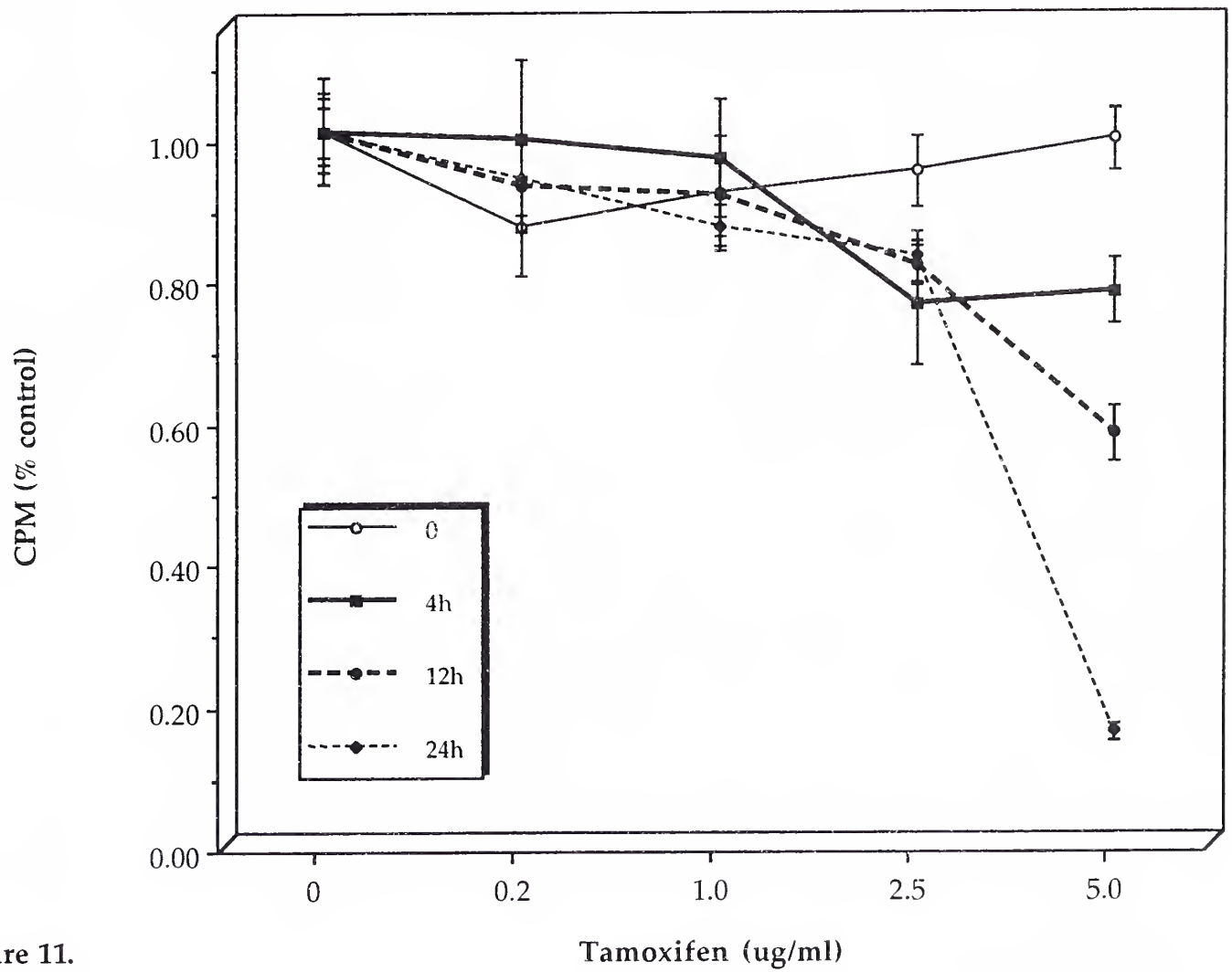


Figure 11.

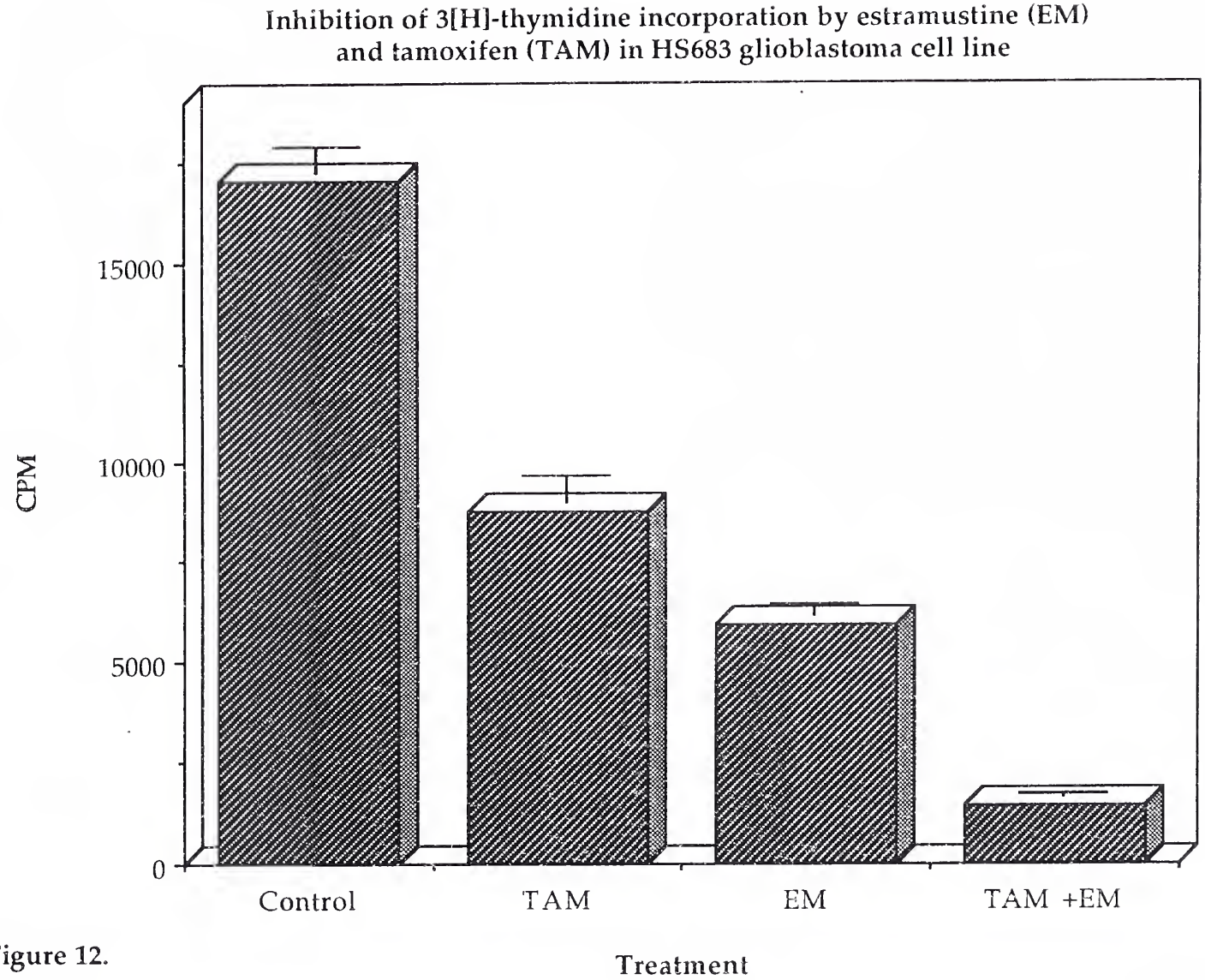

Inhibition of 3[H]-thymidine incorporation by estramustine (EM)
and tamoxifen (TAM) in HS683 glioblastoma cell line
CPM
15000
10000
5000
0
Control
TAM
EM
TAM +EM
Treatment

Figure 12.

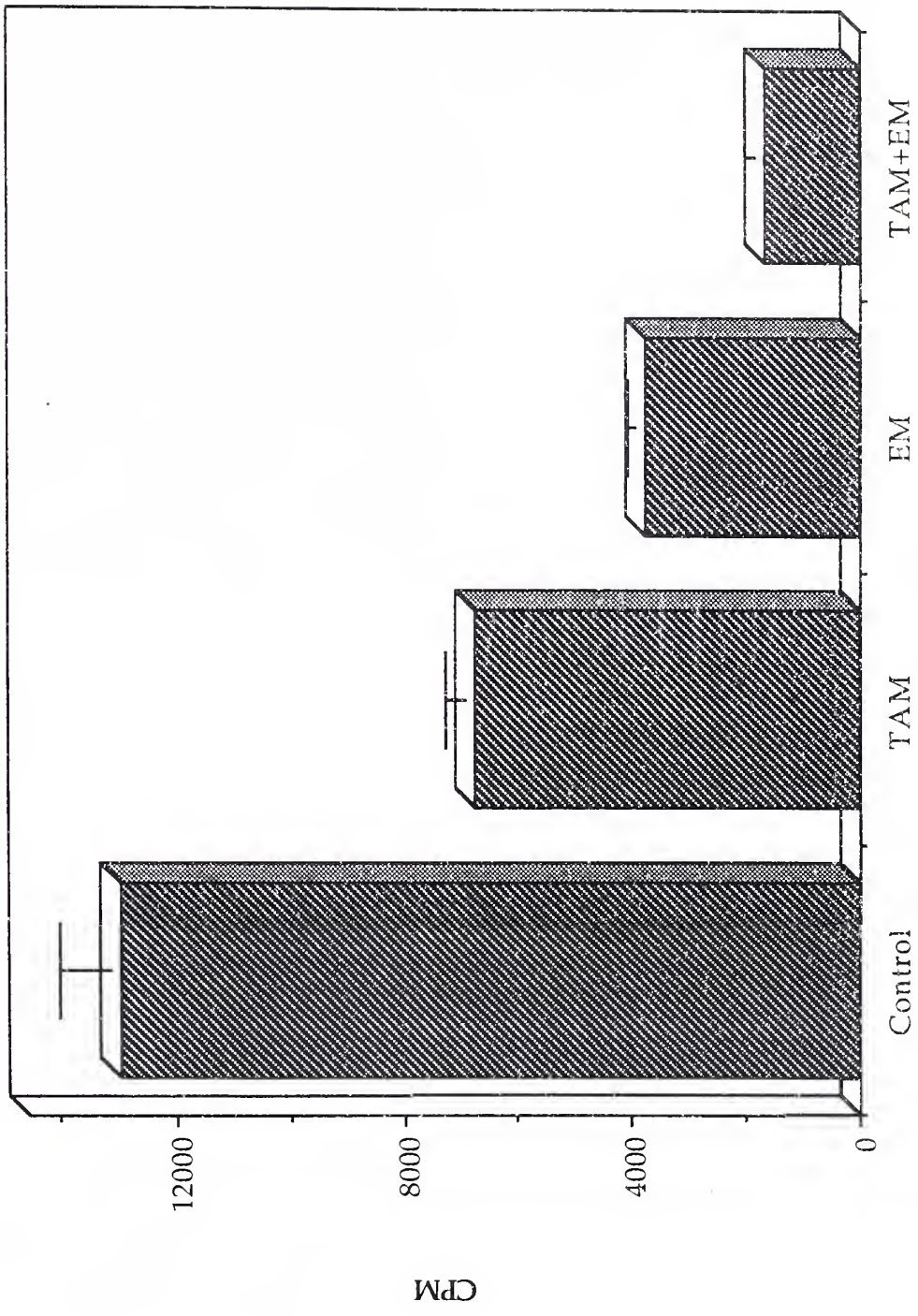

Inhibition of 3[H]-thymidine incorporation in J889H glioblastoma cells by estramustine (EM) and tamoxifen (TAM)
CPM
12000
8000
4000
0
Control
TAM
EM
TAM+EM
Treatment

Figure 13.

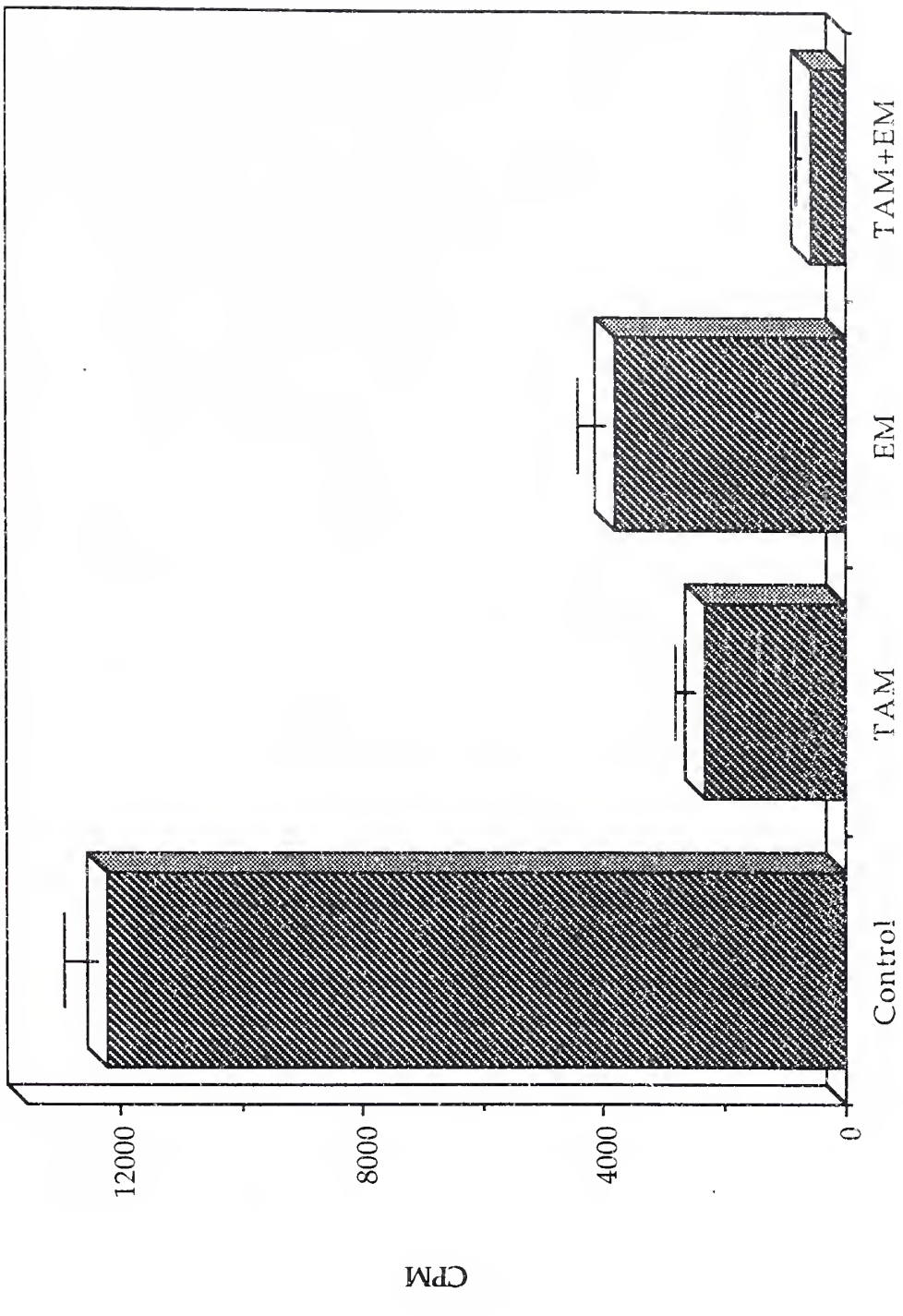

Inhibitionof 3[H]-thymidine incorporation in SZ glioblastoma cells by estramustine (EM) and tamoxifen (TAM)
CPM
12000
8000
4000
0
Control
TAM
EM
TAM+EM
Treatment

Figure 14.

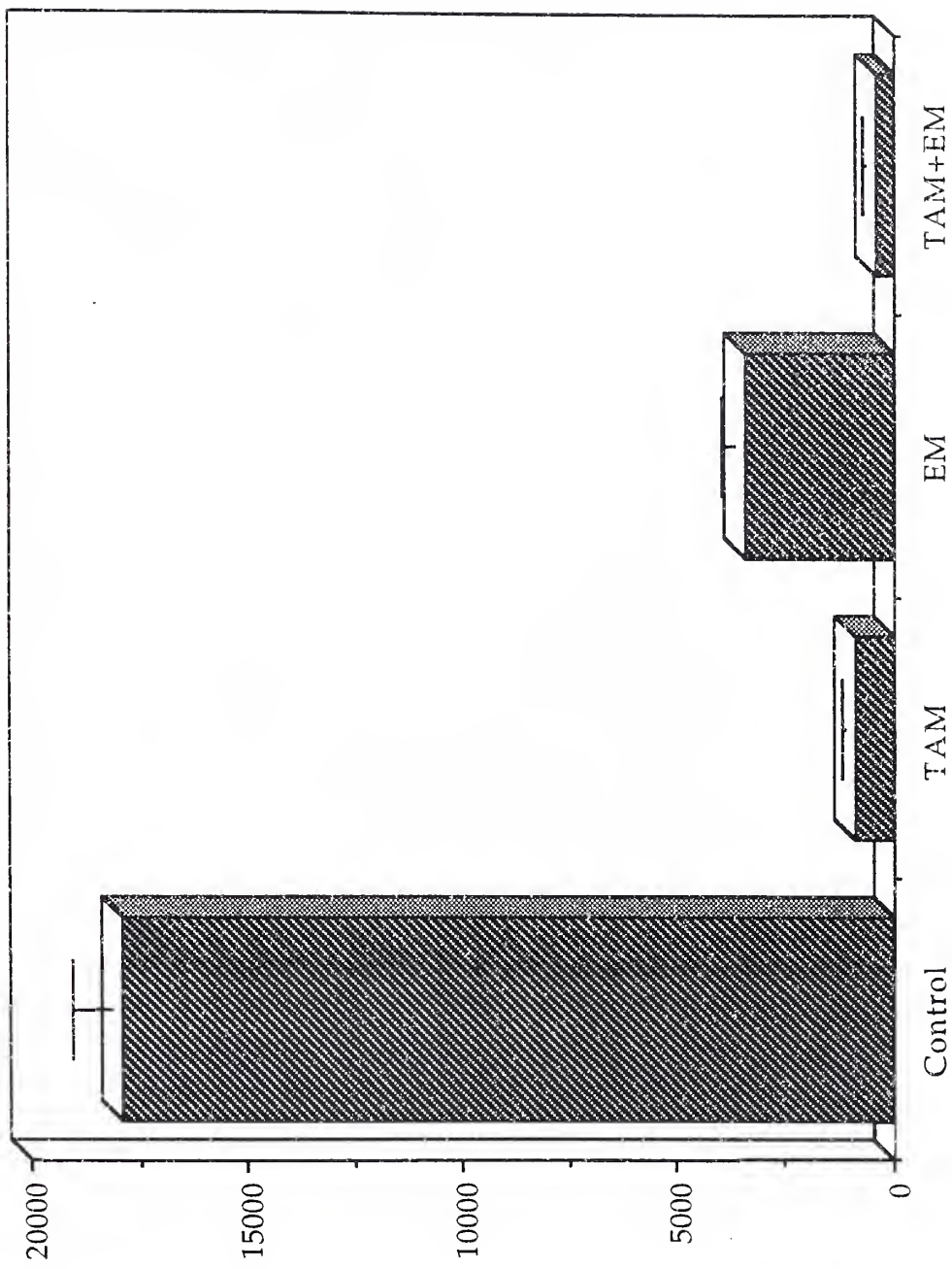

Inhibition of 3[H]-thymidine incorporation in H1289G glioblastoma cells by estramustine (EM) and tamoxifen (TAM)
CPM
20000
15000
10000
5000
0
Control
TAM
EM
TAM+EM
Treatment

Figure 15.

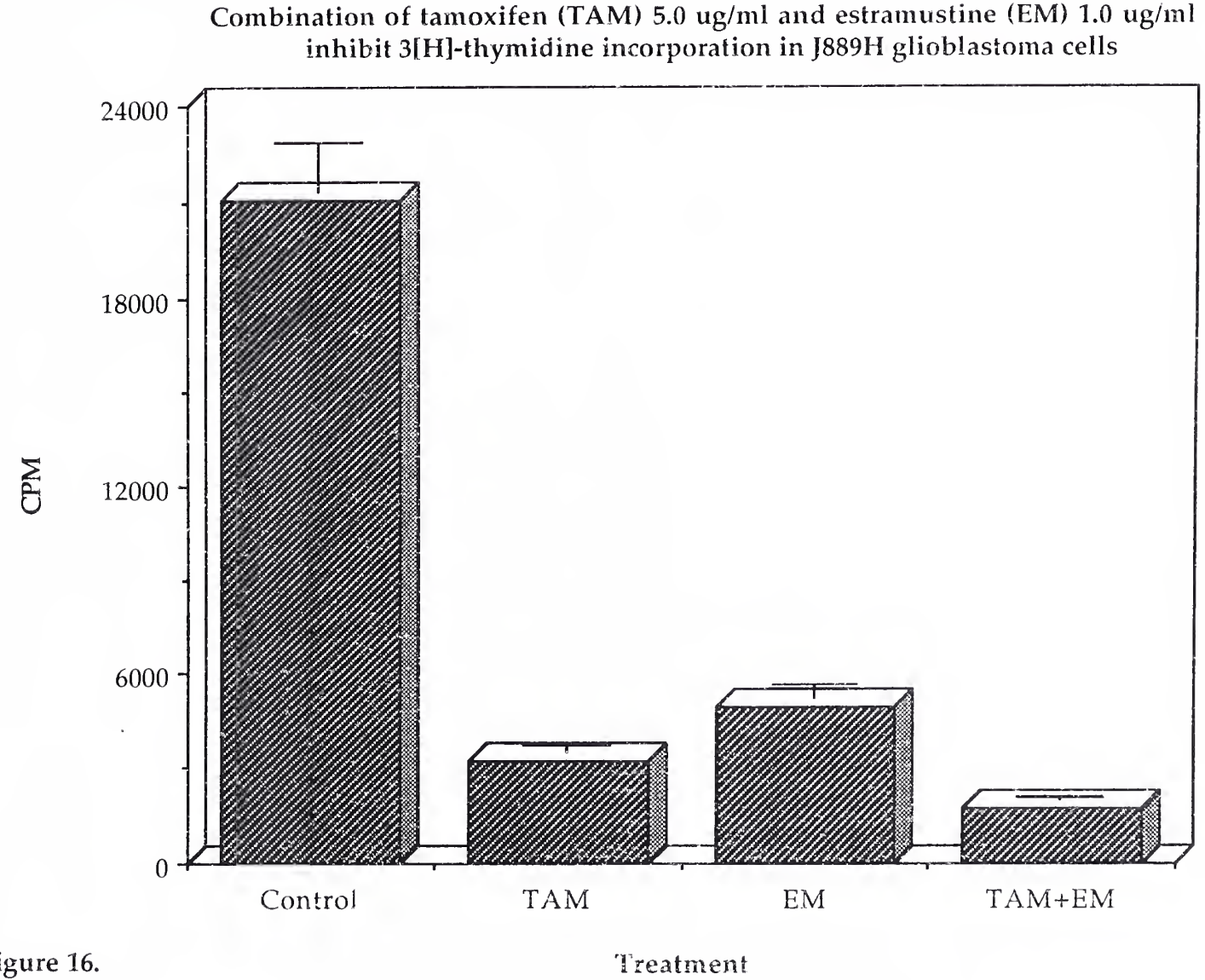

Combination of tamoxifen (TAM) 5.0 ug/ml and estramustine (EM) 1.0 ug/ml
inhibit 3[H]-thymidine incorporation in J889H glioblastoma cells
24000
18000
12000
6000
0
CPM
Control
TAM
EM
TAM+EM
Treatment

Figure 16.

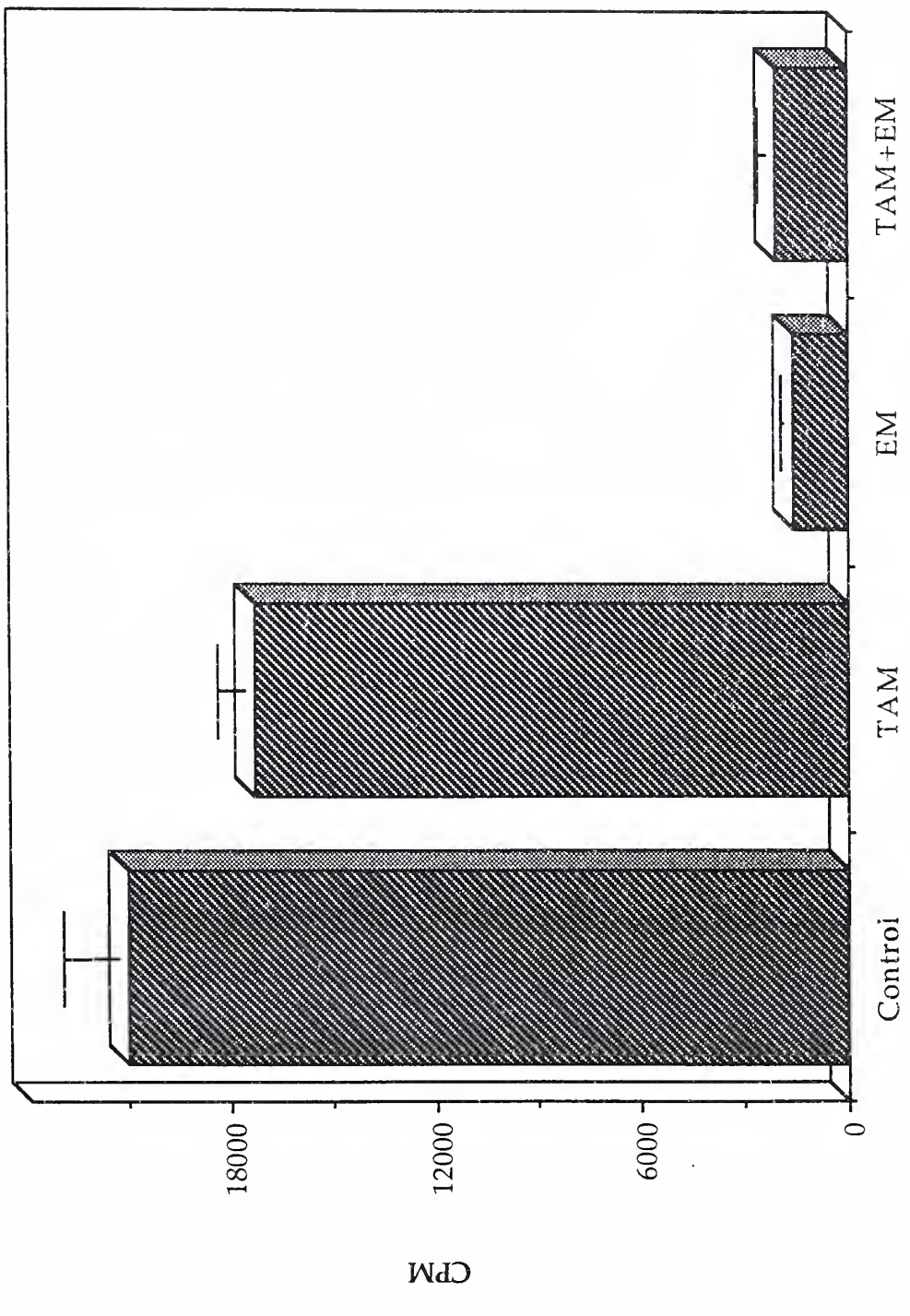

Tamoxifen at a concentration of 2.5 ug/ml does not potentiate the effects of EM on the inhibition of 3[H]-thymidine incorporation in J889H glioblastoma cells
CPM
18000
12000
6000
0
Controi
TAM
EM
TAM+EM
Treatment

Figure 17.

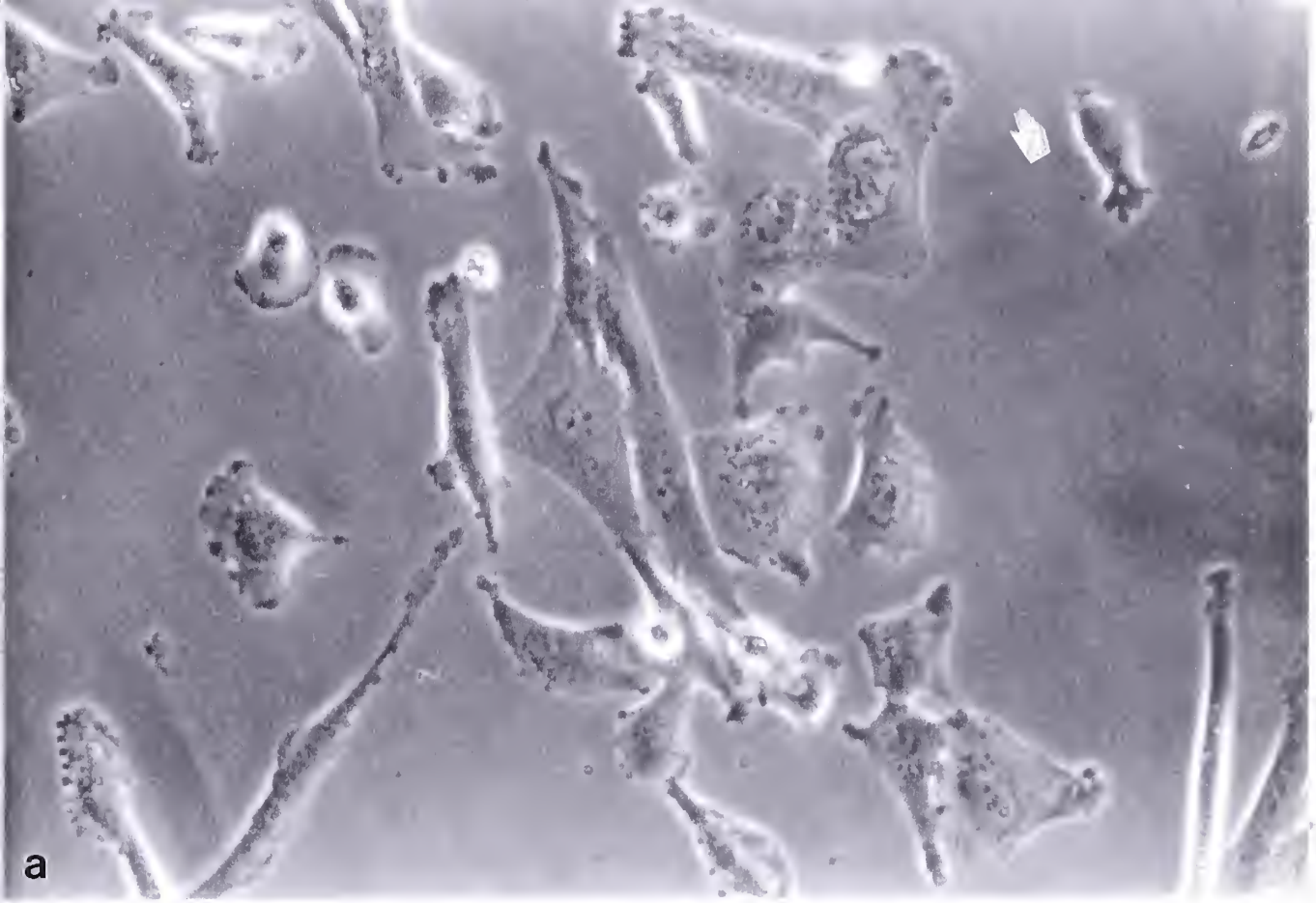


Figure 18a.

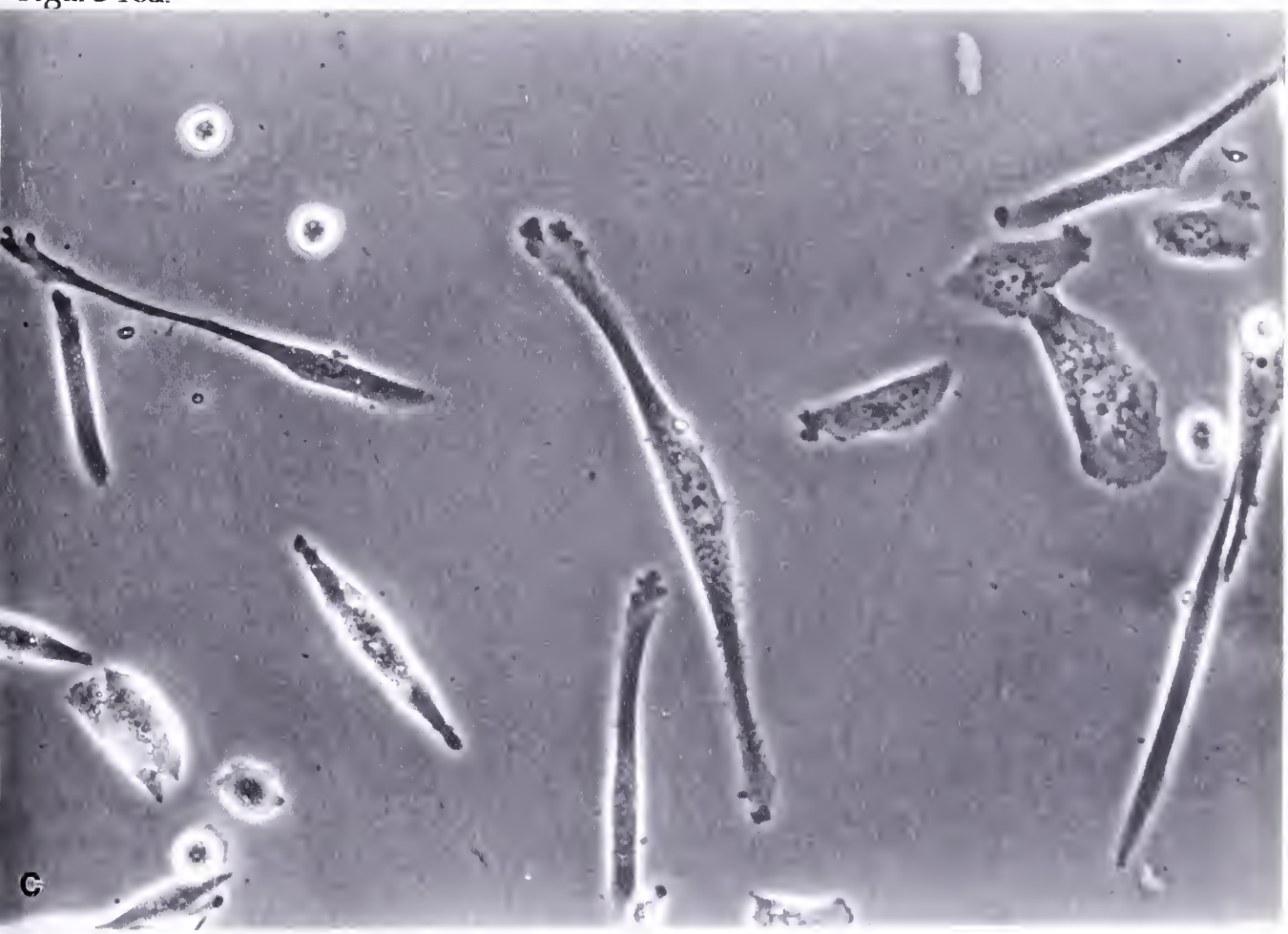


Figure 18b.

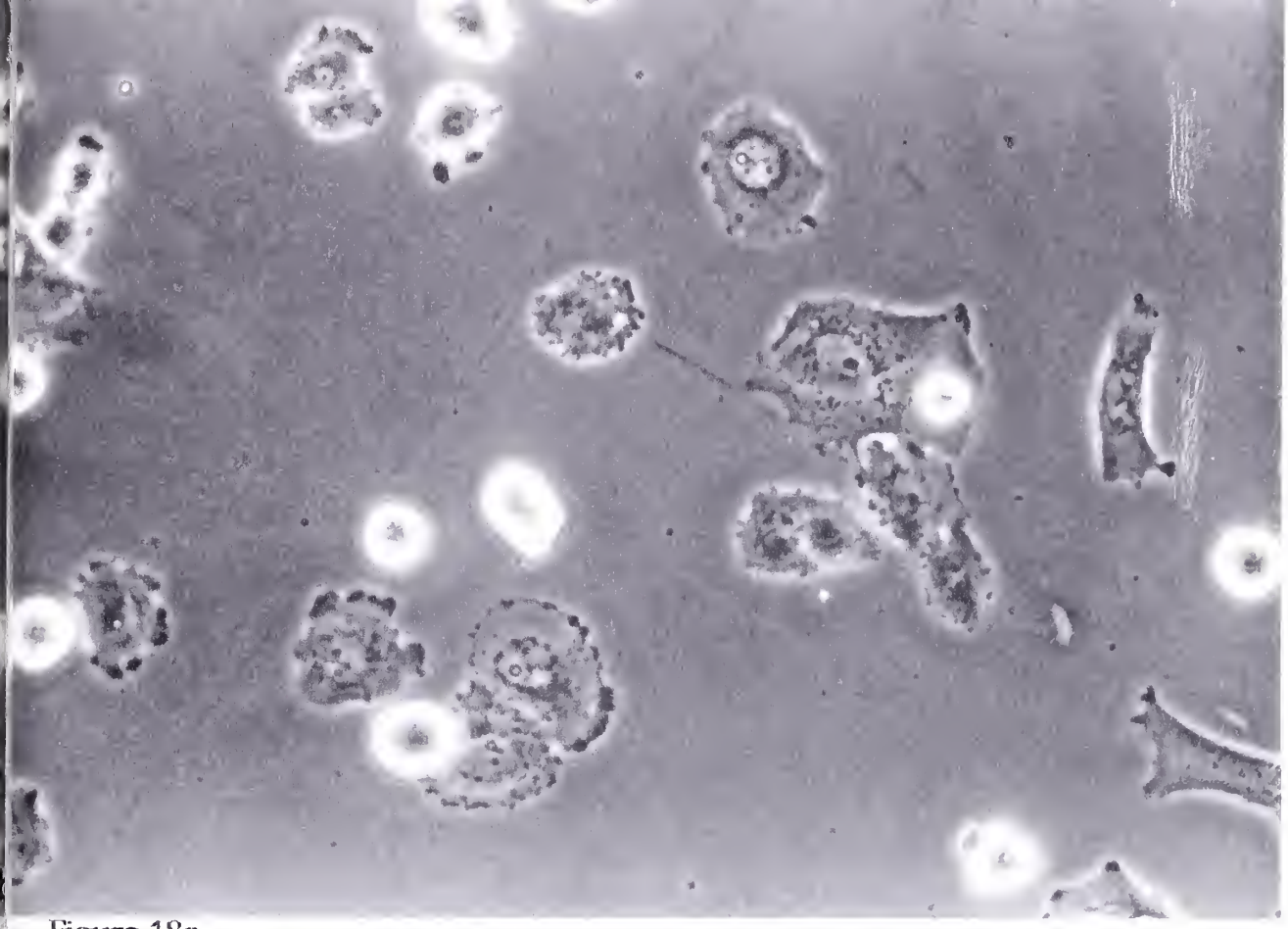


Figure 18c.

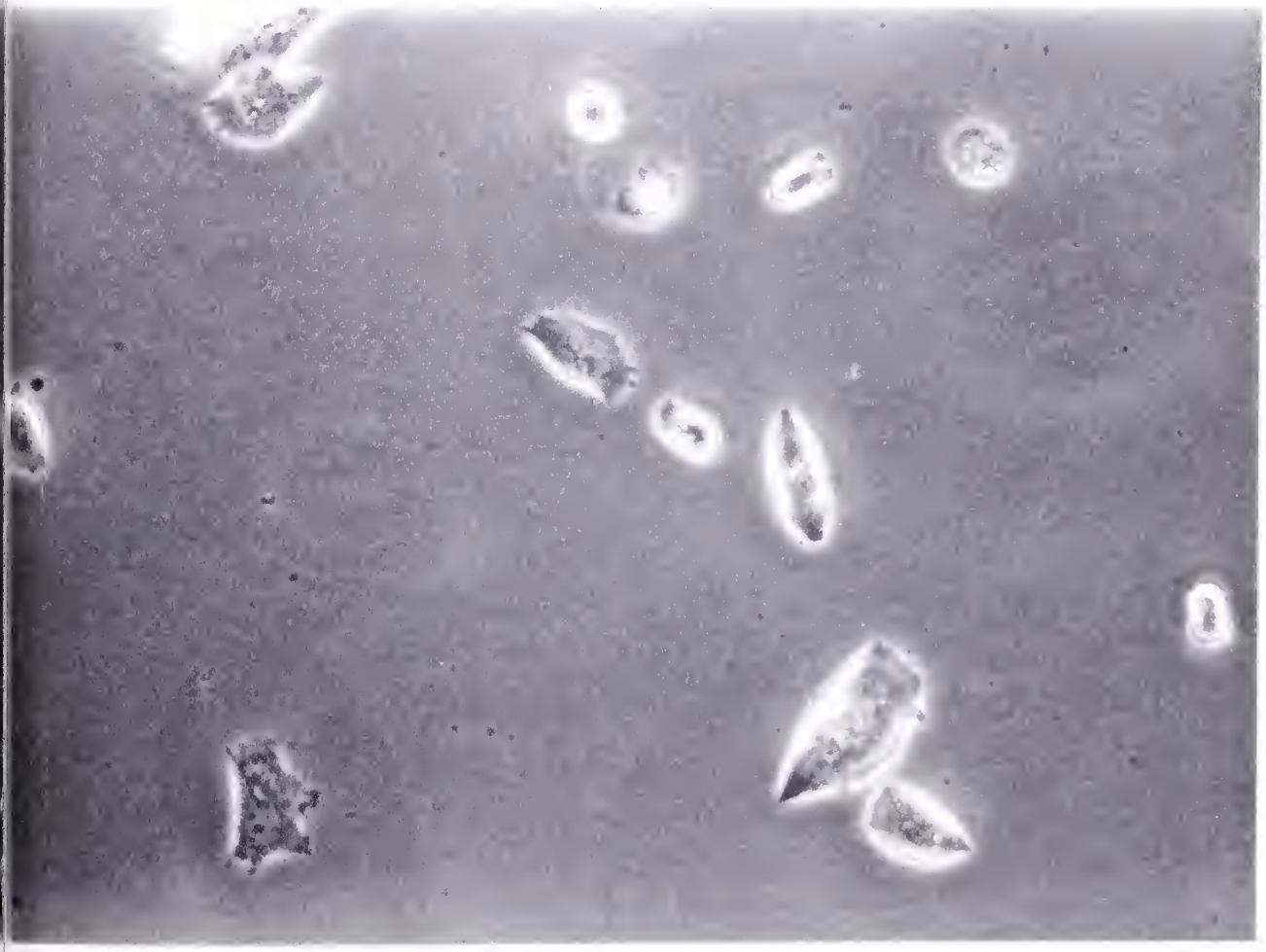


Figure 18d.

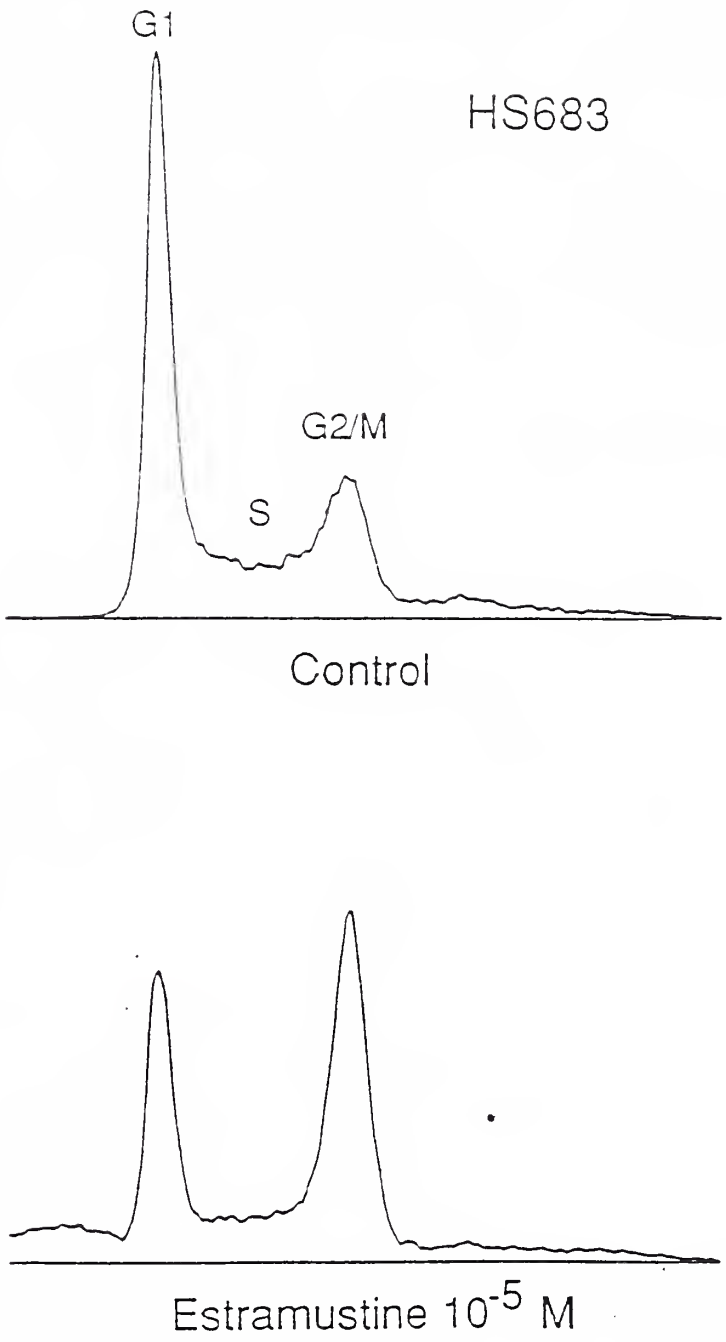


Figure 19a.

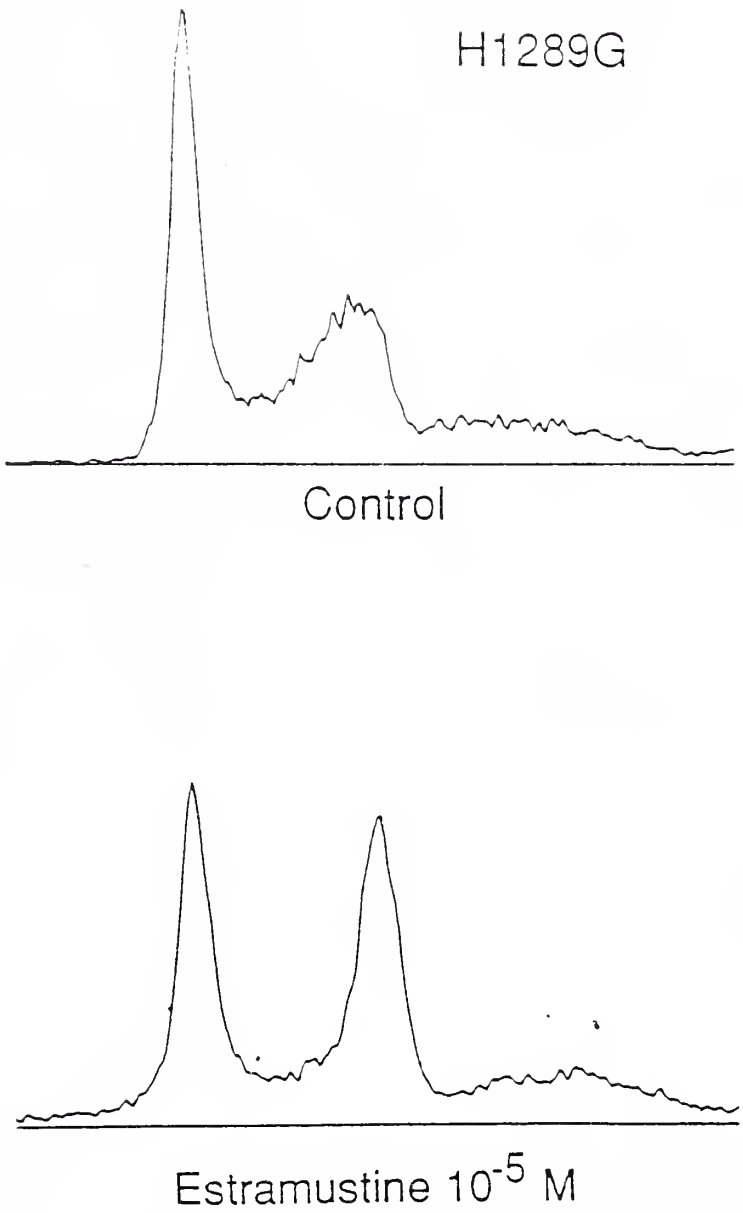


Figure 19b.

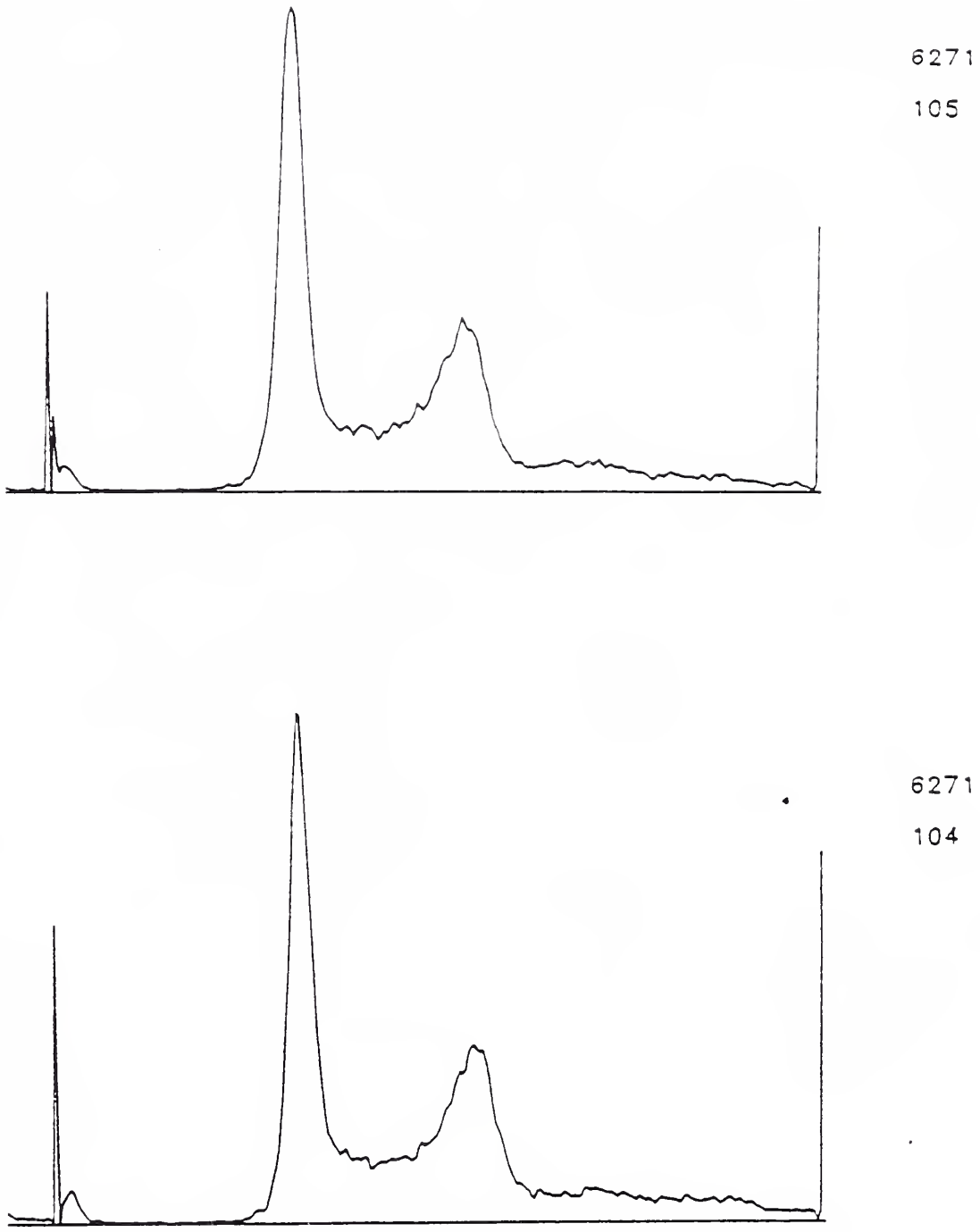
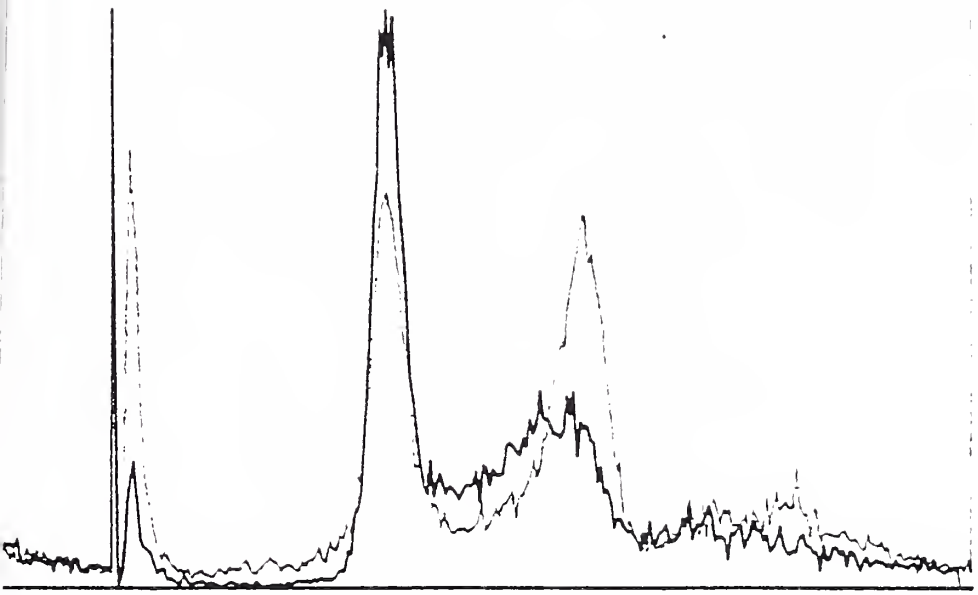


Figure 20.



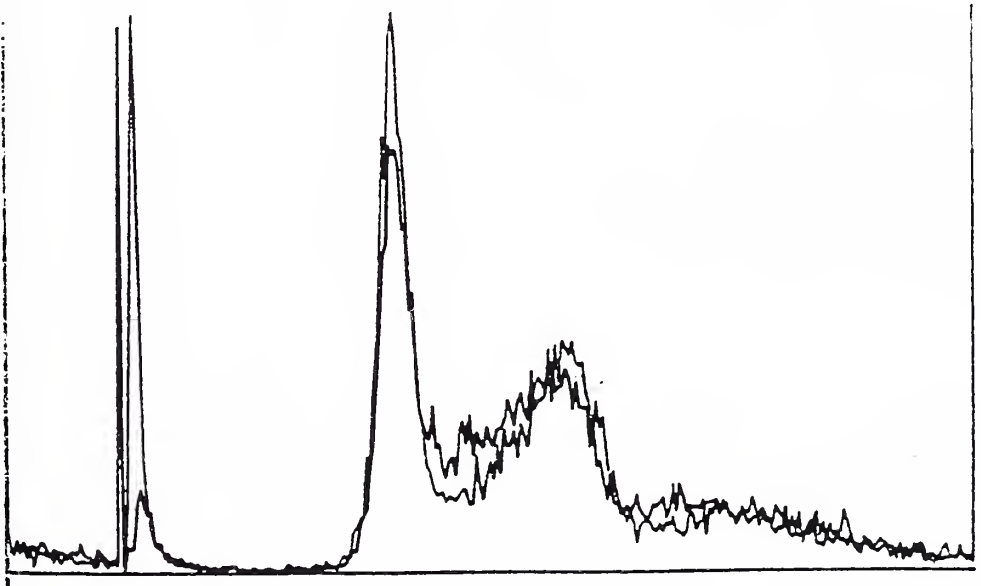


Figure 21.

Effect of increasing concentrations of bleomycin (BLM) on 3H-thymidine incorporation in J889H glioblastoma cells after 4 and 24 hours

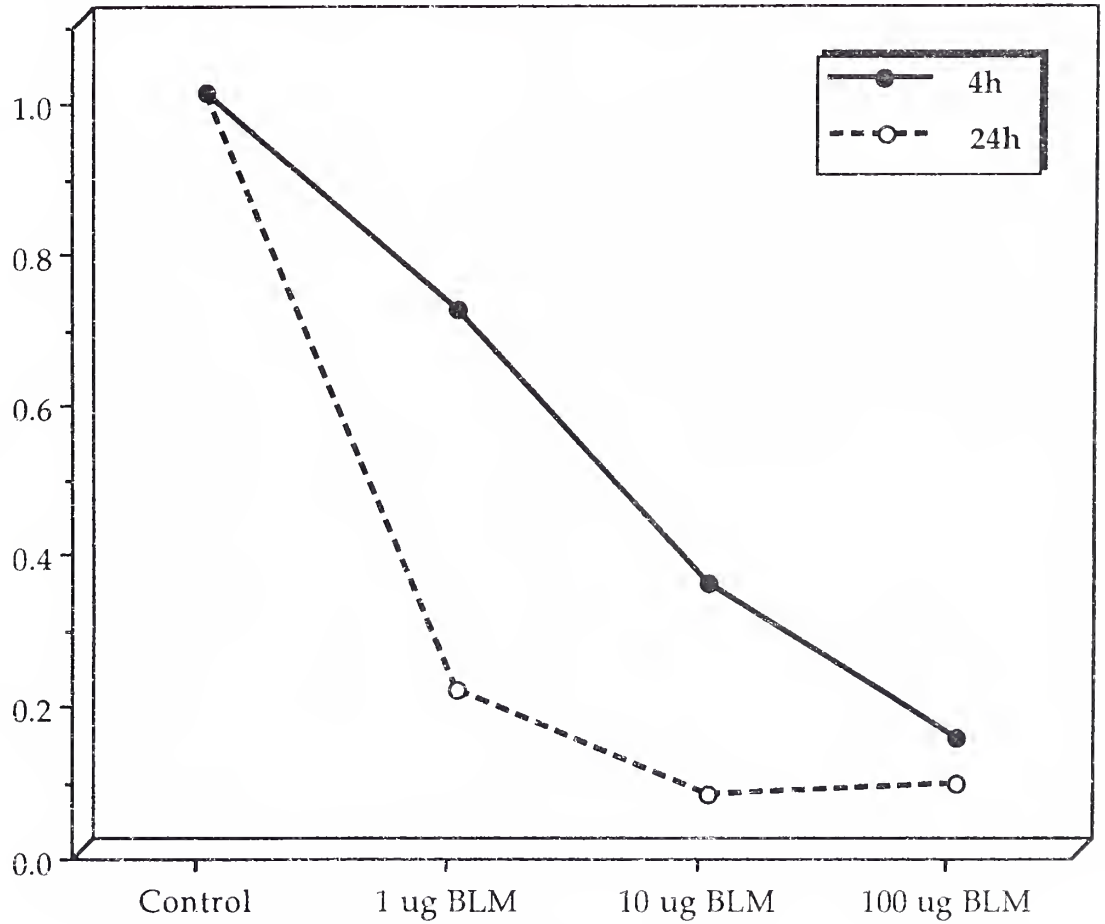


Figure 22.

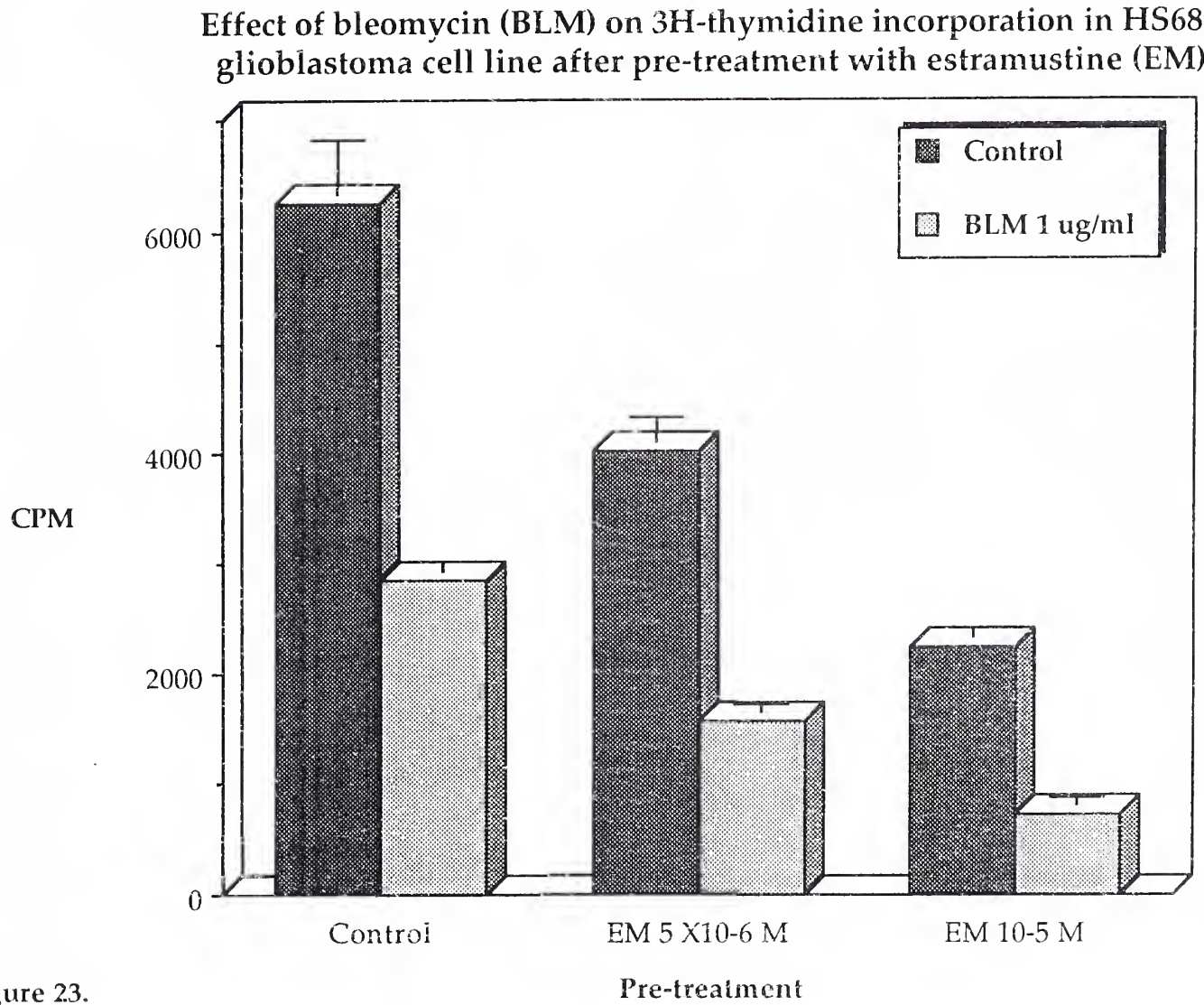

Effect of bleomycin (BLM) on 3H-thymidine incorporation in HS683 glioblastoma cell line after pre-treatment with estramustine (EM)
Control
BLM 1 ug/ml
CPM
6000
4000
2000
0
Control
EM 5 X10-6 M
EM 10-5 M
Pre-treatment

Figure 23.

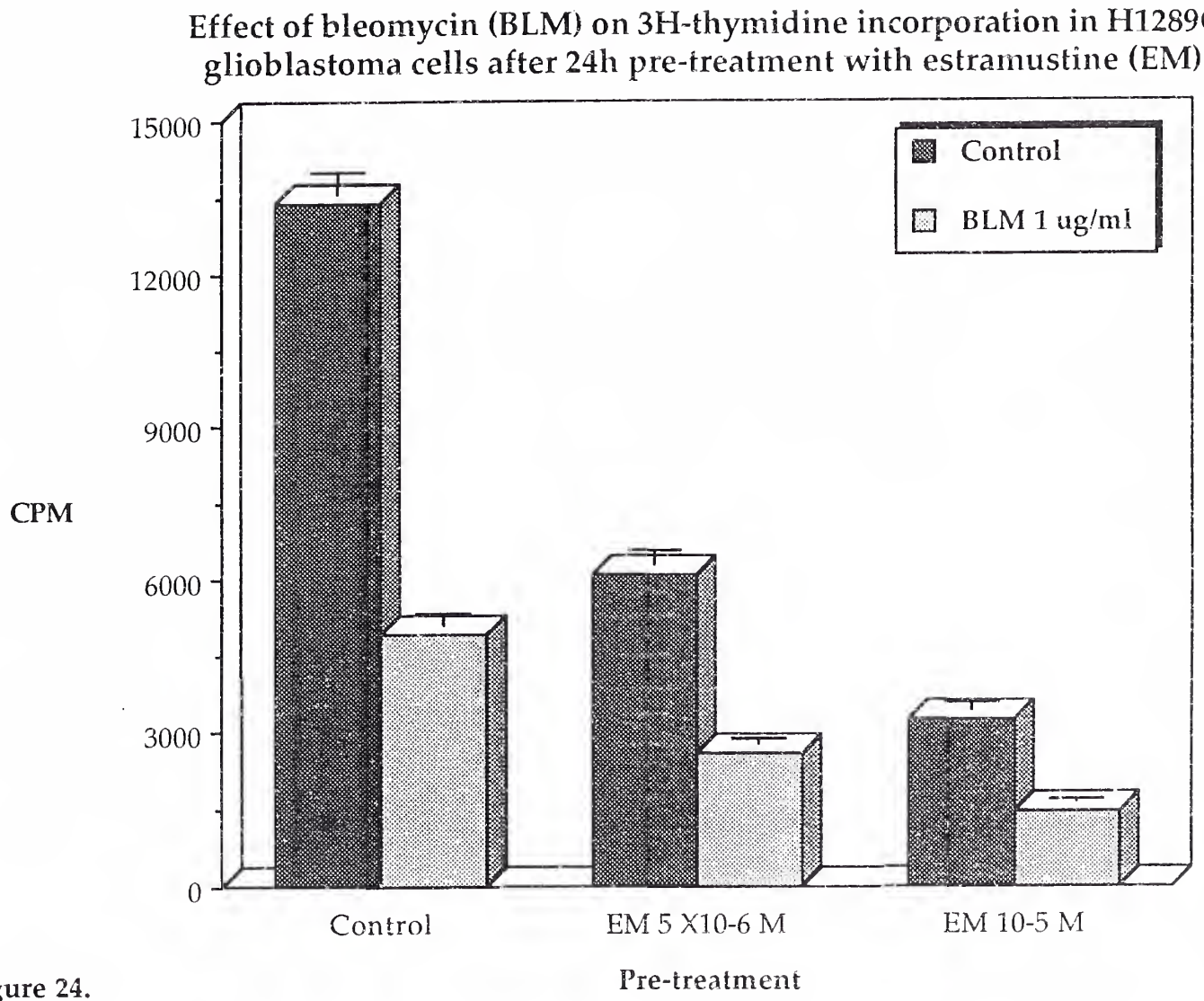

Effect of bleomycin (BLM) on 3H-thymidine incorporation in H1289G glioblastoma cells after 24h pre-treatment with estramustine (EM)
15000
12000
9000
6000
3000
0
CPM
Control
BLM 1 ug/ml
Control
EM 5 X10-6 M
EM 10-5 M
Pre-treatment

Figure 24.

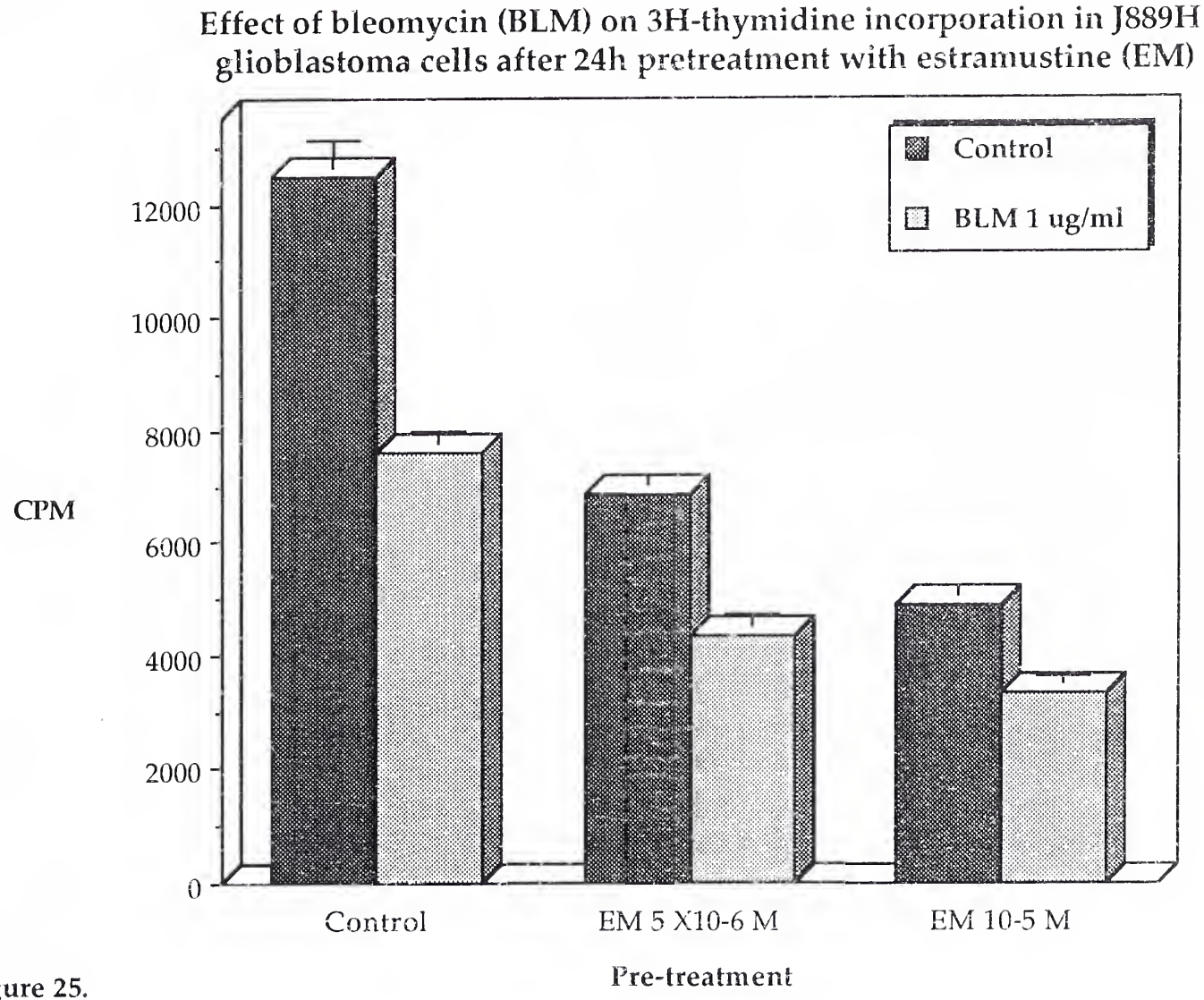

Effect of bleomycin (BLM) on 3H-thymidine incorporation in J889H glioblastoma cells after 24h pretreatment with estramustine (EM)
Control
BLM 1 ug/ml
CPM
12000
10000
8000
6000
4000
2000
0
Control
EM 5 X10-6 M
EM 10-5 M
Pre-treatment

Figure 25.

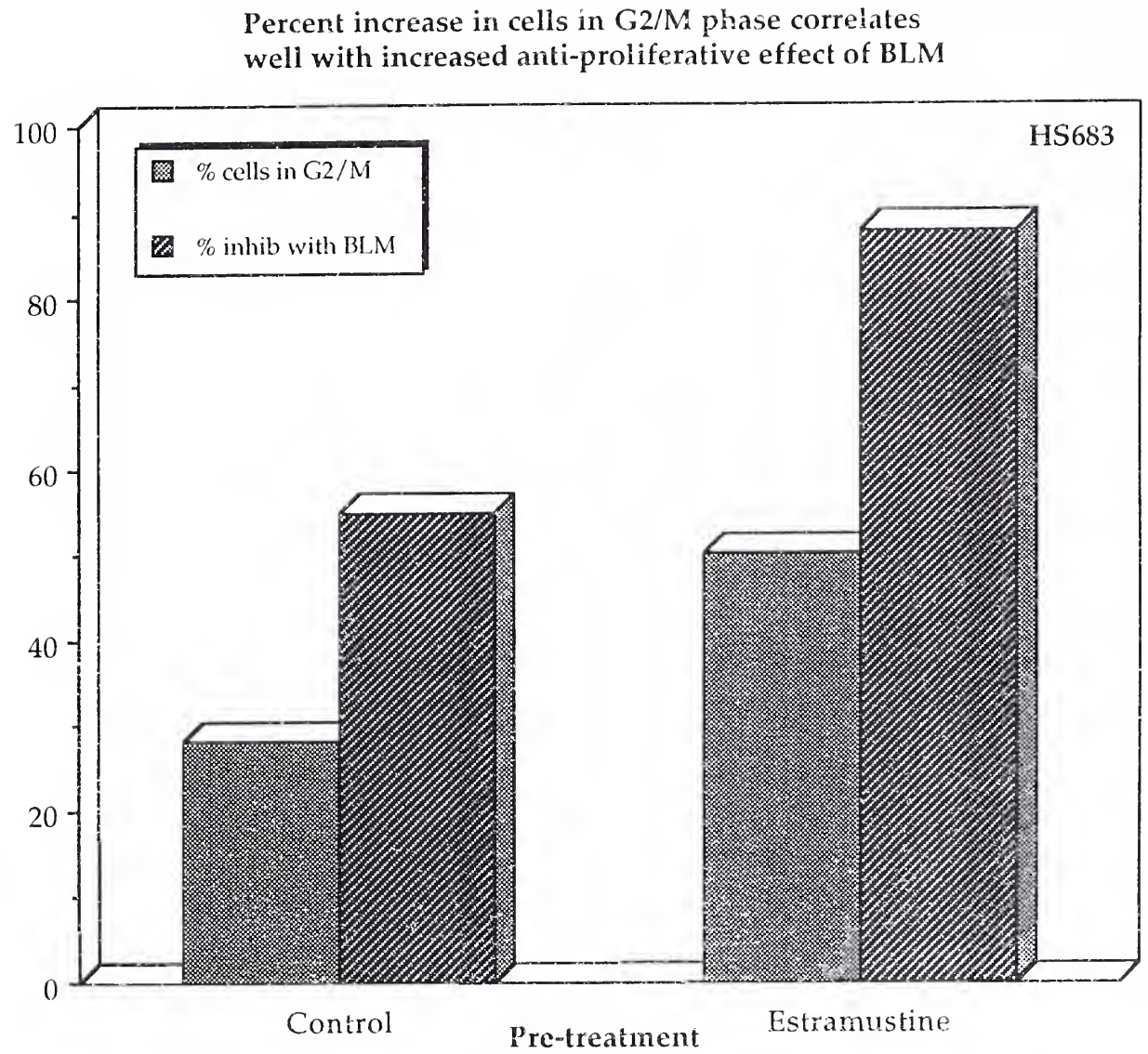

Percent increase in cells in G2/M phase correlates well with increased anti-proliferative effect of BLM
HS683
% cells in G2/M
% inhib with BLM
100
80
60
40
20
0
Control
Pre-treatment
Estramustine

Figure 26.

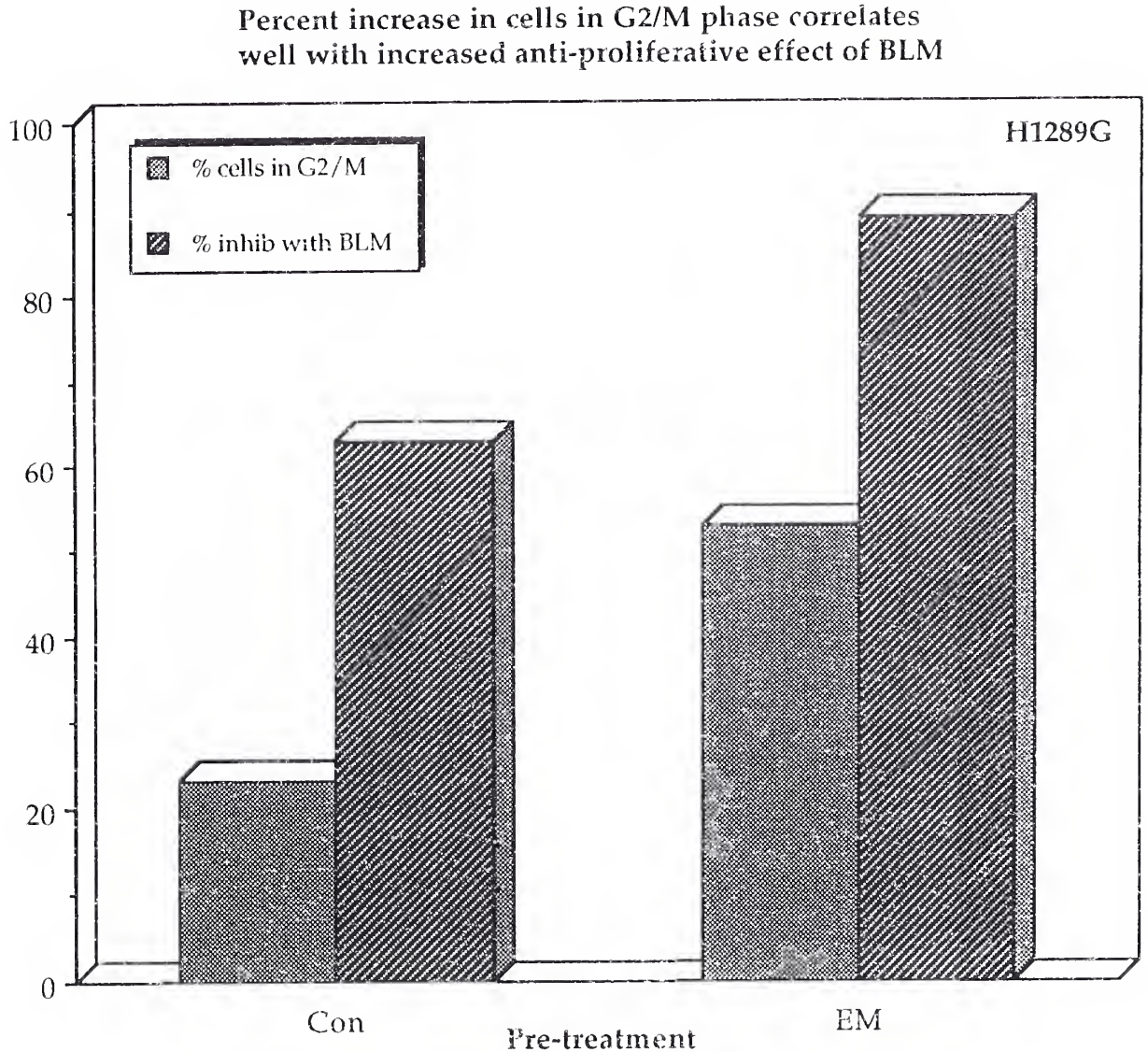

Percent increase in cells in G2/M phase correlates
well with increased anti-proliferative effect of BLM
H1289G
% cells in G2/M
% inhib with BLM
100
80
60
40
20
0
Con
EM
Pre-treatment

Figure 27.

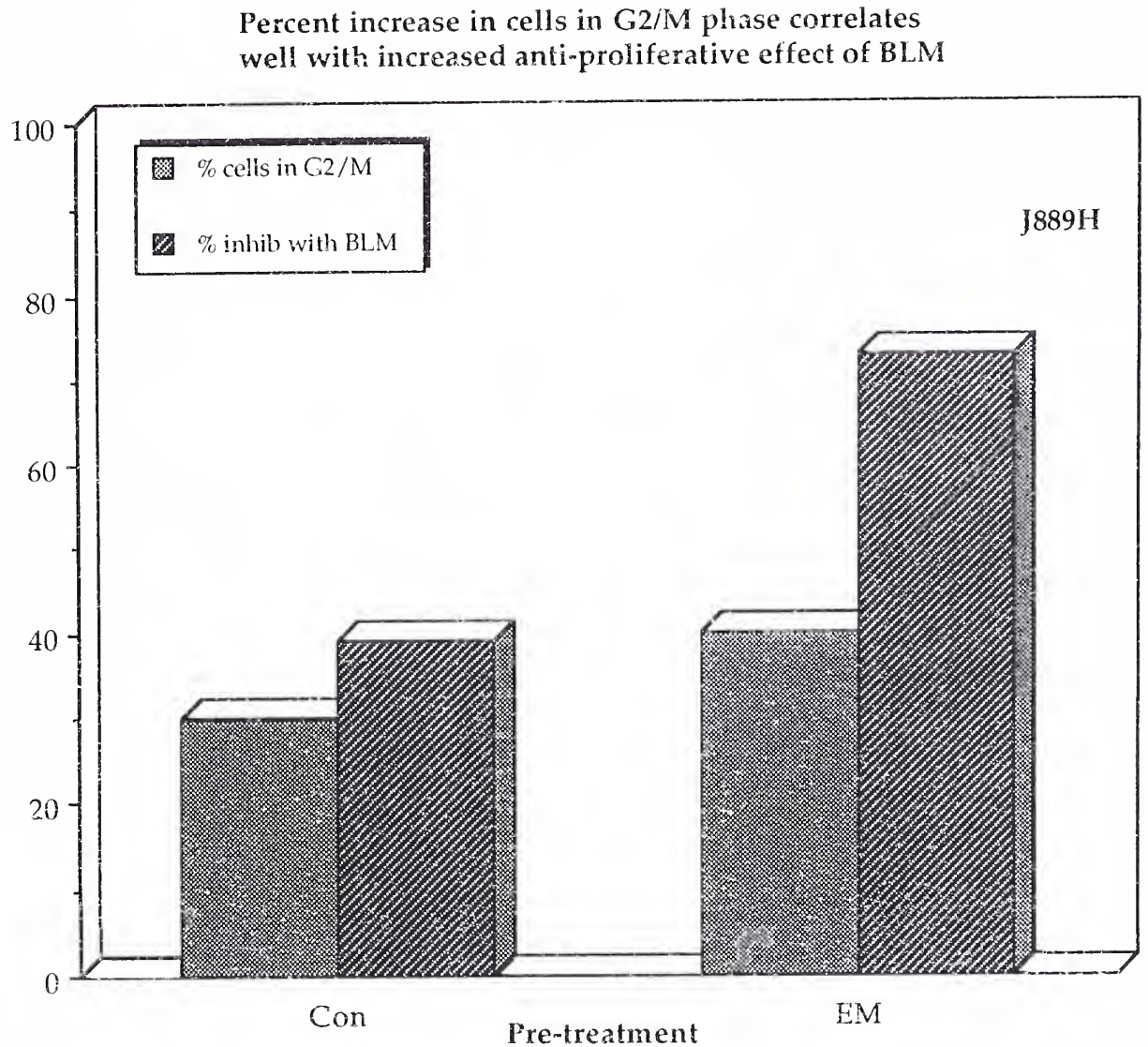

Percent increase in cells in G2/M phase correlates
well with increased anti-proliferative effect of BLM
% cells in G2/M
% inhib with BLM
J889H
100
80
60
40
20
0
Con
EM
Pre-treatment

Figure 28.

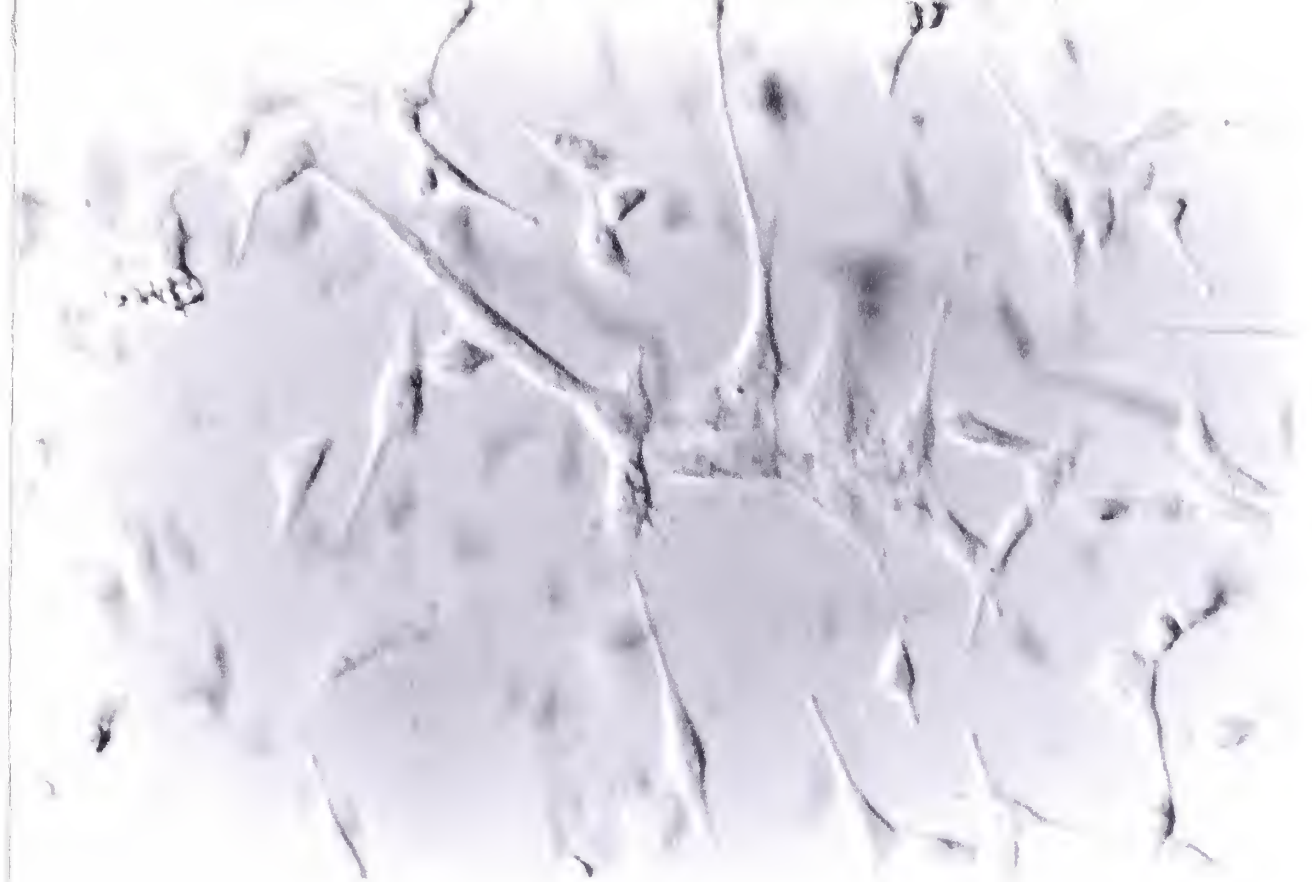


Figure 29a.

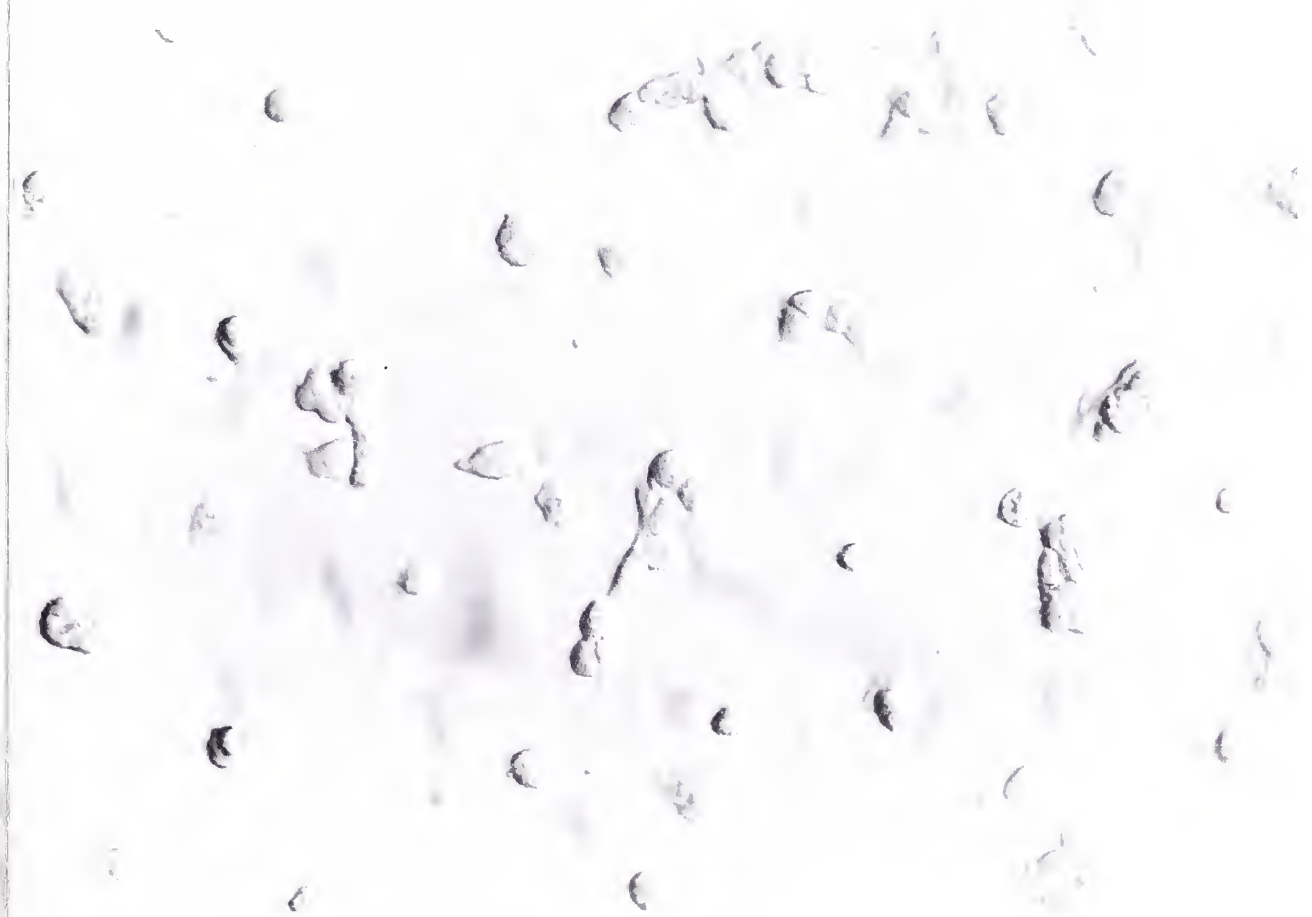


Figure 29b.

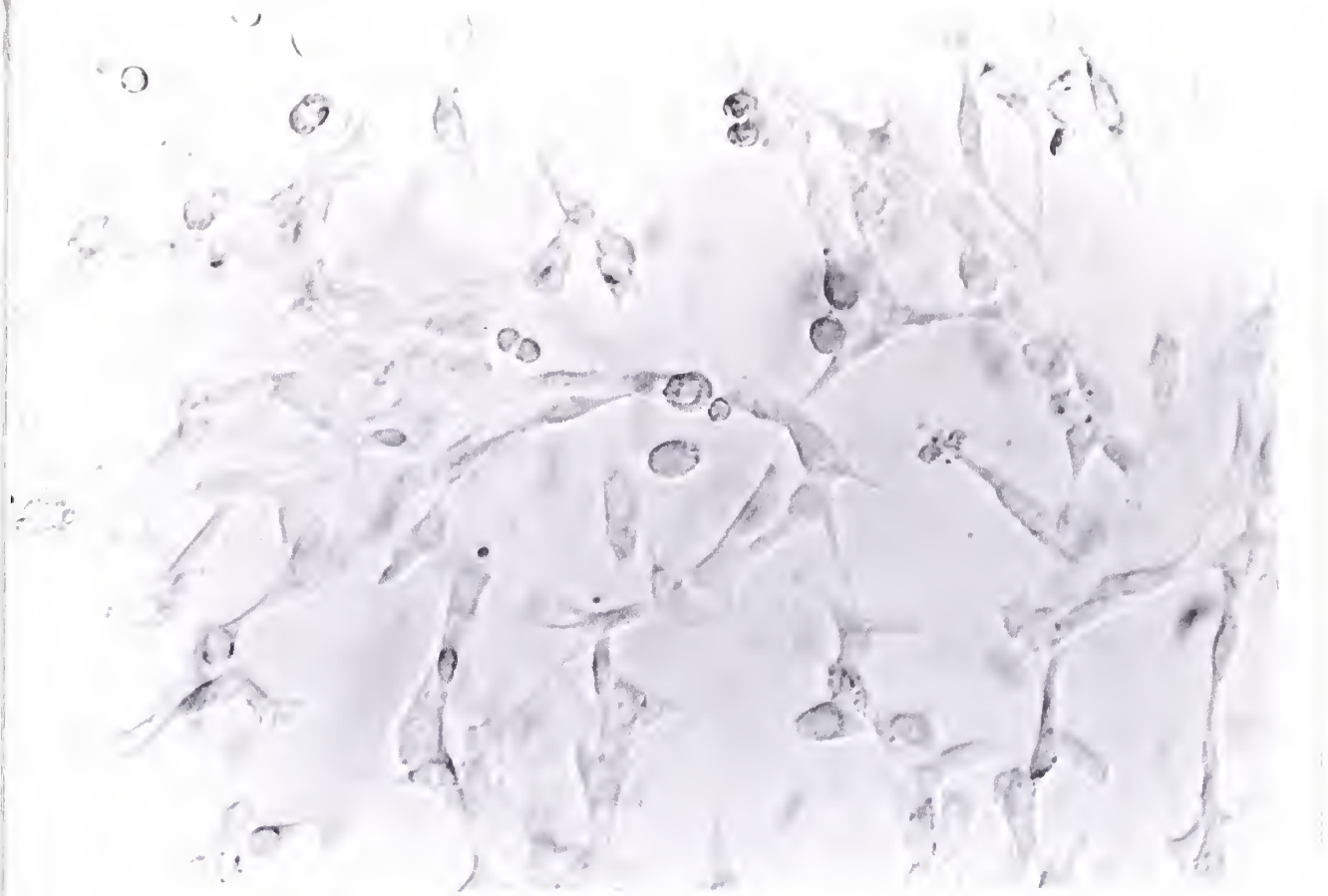


Figure 29c.

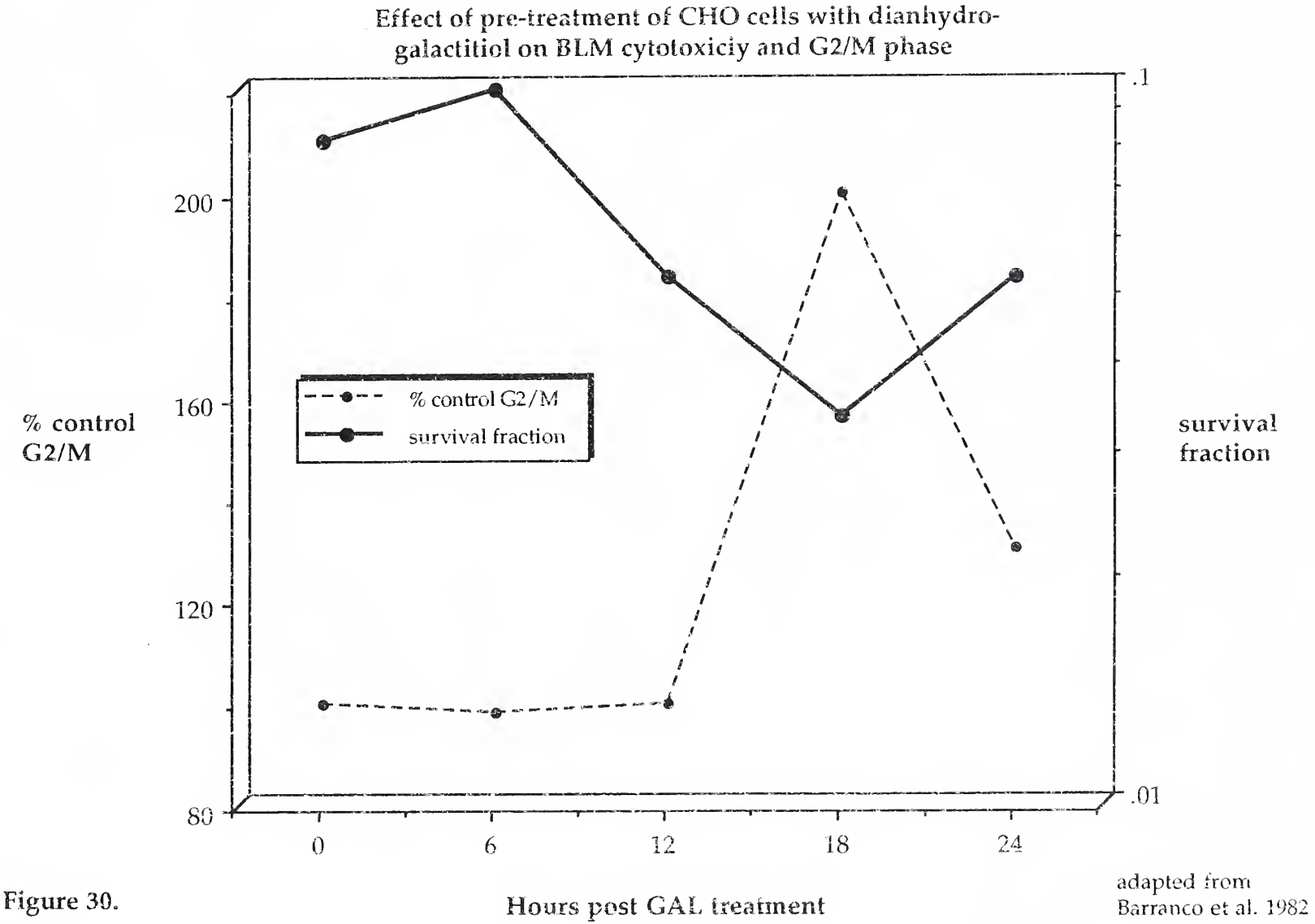


Figure 30.

adapted from Barranco et al. 1982

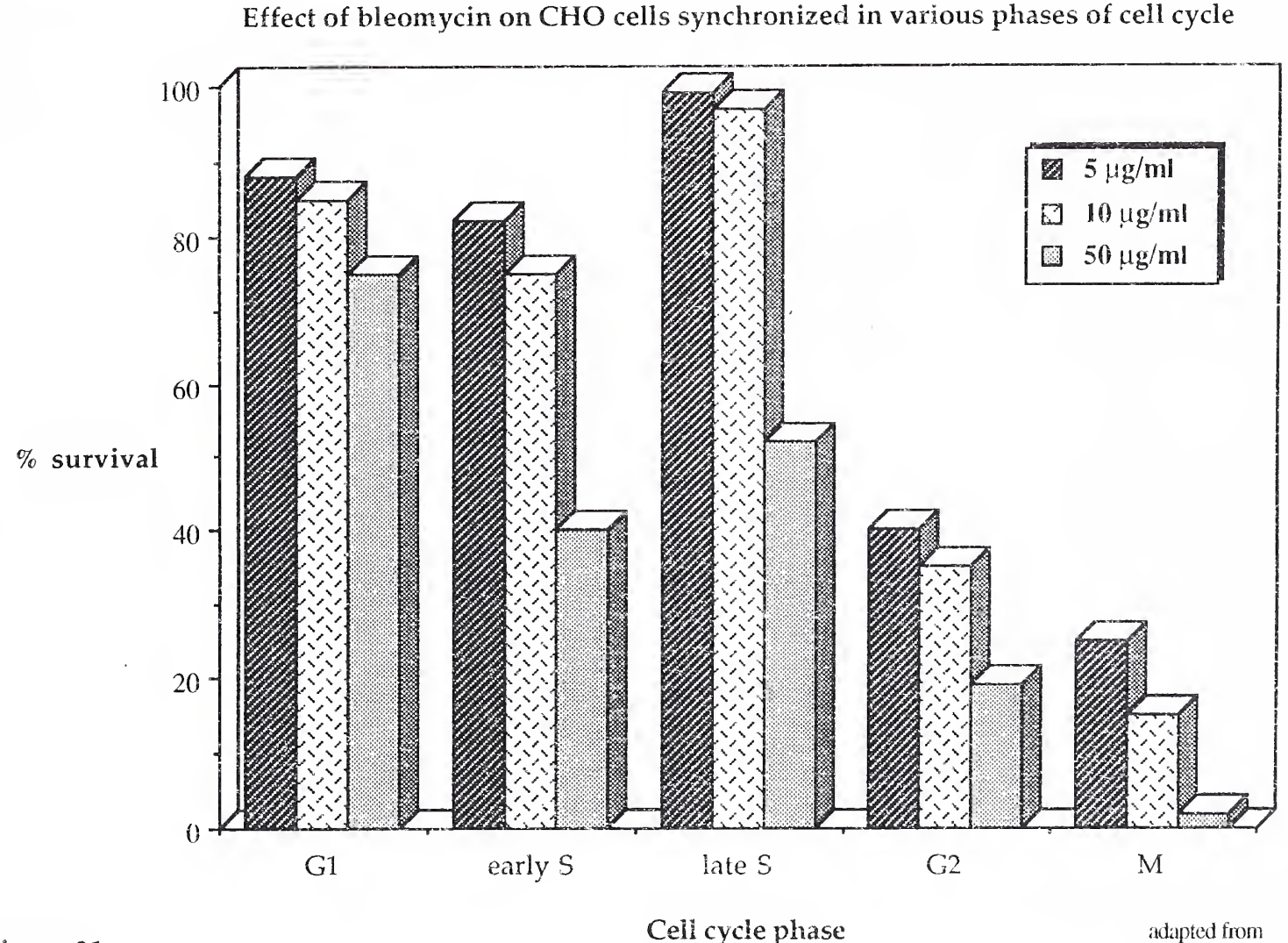


Figure 31.

adapted from
Barranco & Humphrey 1971

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